

(86) Date de dépôt PCT/PCT Filing Date: 1993/12/02
(87) Date publication PCT/PCT Publication Date: 1994/06/09
(45) Date de délivrance/Issue Date: 2008/05/27
(85) Entrée phase nationale/National Entry: 1995/03/27
(86) N° demande PCT/PCT Application No.: US 1993/011667
(87) N° publication PCT/PCT Publication No.: 1994/012649
(30) Priorités/Priorities: 1992/12/03 (US07/985,478);
1993/10/01 (US08/130,682); 1993/10/13 (US08/136,742)

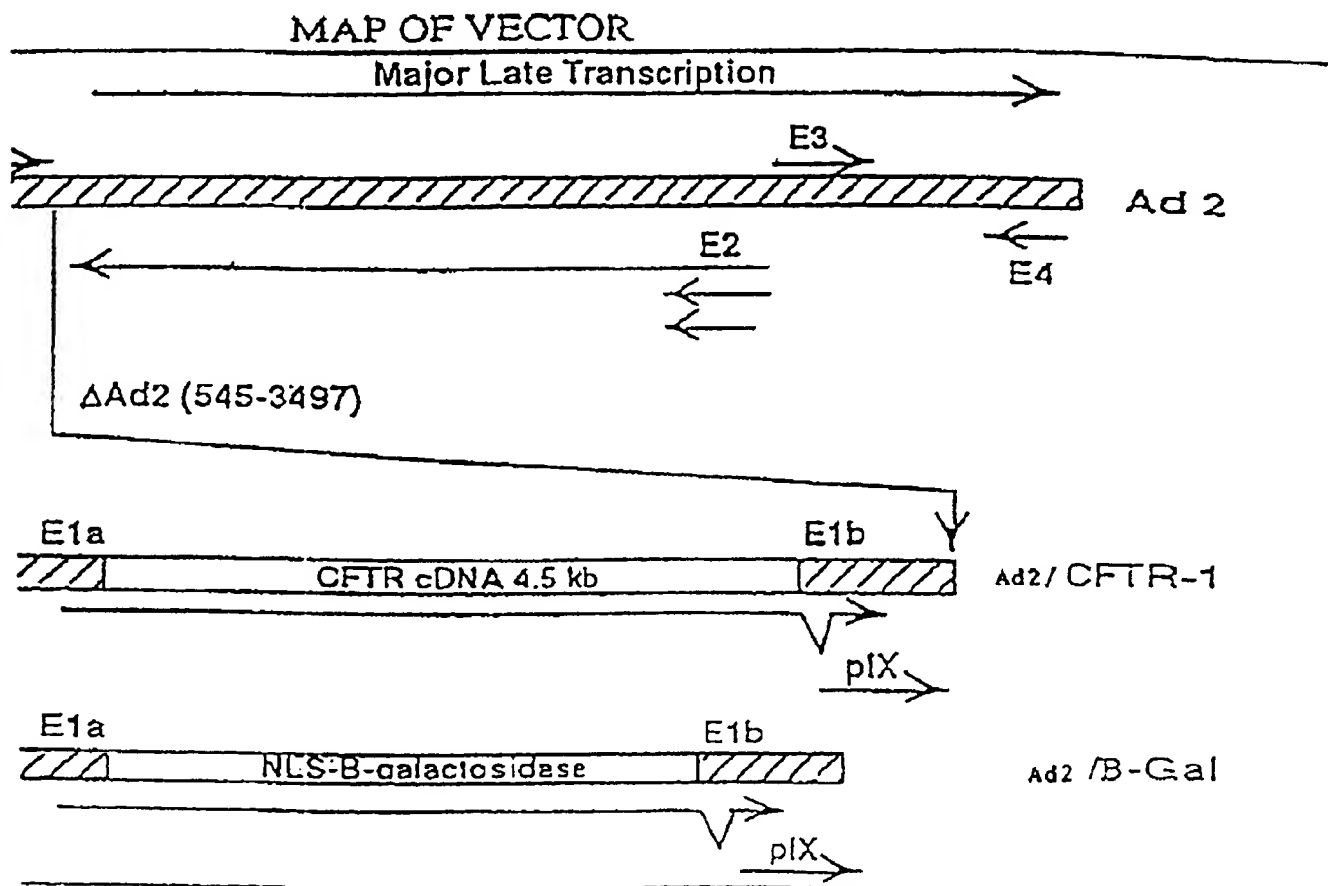
(51) Cl.Int./Int.Cl. *C12N 15/861* (2006.01),
A61K 38/00 (2006.01), *A61K 48/00* (2006.01),
C07K 14/755 (2006.01), *C12N 15/86* (2006.01),
C12N 9/64 (2006.01)

(72) Inventeurs/Inventors:
GREGORY, RICHARD J., US;
ARMENTANO, DONNA, US;
COUTURE, LARRY A., US;
SMITH, ALAN E., US

(73) Propriétaire/Owner:
GENZYME CORPORATION, US

(74) Agent: SMART & BIGGAR

(54) Titre : VECTEURS PSEUDO-ADENOVIRAUX
(54) Title: PSEUDO-ADENOVIRUS VECTORS



(57) Abrégé/Abstract:

Gene Therapy vectors, which are especially useful for cystic fibrosis, and methods for using the vectors are disclosed. In preferred embodiments, the vectors are adenovirus-based. Advantages of adenovirus-based vectors for gene therapy are that they appear to

(57) Abrégé(suite)/Abstract(continued):

be relatively safe and can be manipulated to encode the desired gene product and at the same time are inactivated in terms of their ability to replicate in a normal lytic viral life cycle. Additionally, adenovirus has a natural tropism for airway epithelia. Therefore, adenovirus-based vectors are particularly preferred for respiratory gene therapy applications such as gene therapy for cystic fibrosis. In one embodiment, the adenovirus-based gene therapy vector comprises an adenovirus 2 serotype genome in which the E1a and E1b regions of the genome, which are involved in early stages of viral replication have been deleted and replaced by genetic material of interest (e.g., DNA encoding the cystic fibrosis transmembrane regulator protein). In another embodiment, the adenovirus-based therapy vector is a pseudo-adenovirus (PAV). PAVs contain no potentially harmful viral genes, have a theoretical capacity for foreign material of nearly 36 kb, may be produced in reasonably high titers and maintain the tropism of the parent adenovirus for dividing and non-dividing human target cell types.



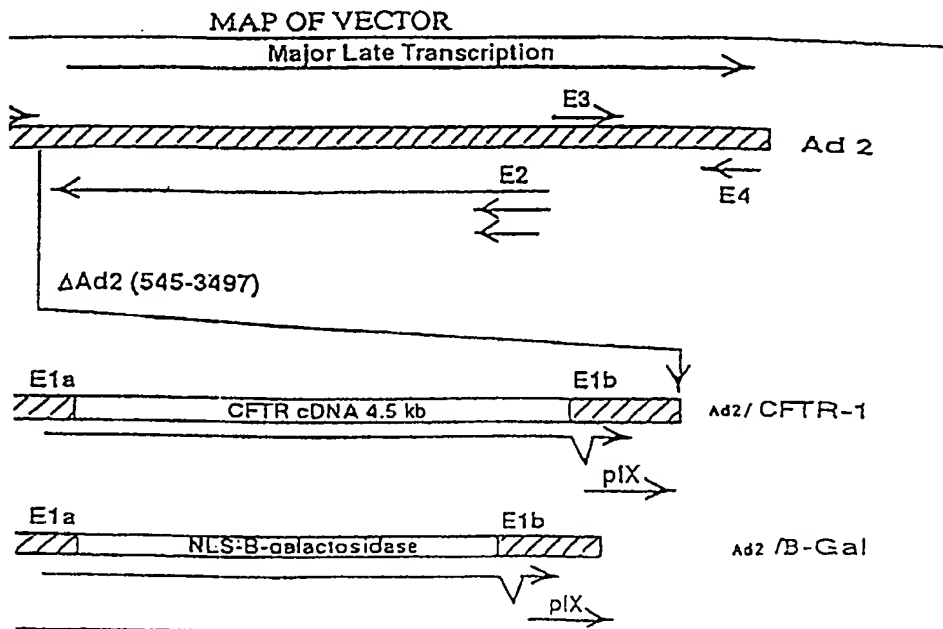
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|
| (51) International Patent Classification ⁵ : C12N 15/86, 15/12, A61K 48/00 | | A3 | (11) International Publication Number: WO 94/12649 |
| | | | (43) International Publication Date: 9 June 1994 (09.06.94) |
| (21) International Application Number: PCT/US93/11667 | | (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). | |
| (22) International Filing Date: 2 December 1993 (02.12.93) | | | |
| (30) Priority Data: 07/985,478 3 December 1992 (03.12.92) US 08/130,682 1 October 1993 (01.10.93) US 08/136,742 13 October 1993 (13.10.93) US | | Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. | |
| (71) Applicant: GENZYME CORPORATION [US/US]; One Kendall Square, Cambridge, MA 02139 (US). | | (88) Date of publication of the international search report: 10 November 1994 (10.11.94) | |
| (72) Inventors: GREGORY, Richard, J.; 4789 Gateshead Road, Carlsbad, CA 92008 (US). ARMENTANO, Donna; 33 Carver Road, Watertown, MA 02172 (US). COUTURE, Larry, A.; 67 Circle Drive, Framingham, MA 01701 (US). SMITH, Alan, E.; 88 Cleveland Road, Wellesley, MA 02181 (US). | | | |
| (74) Agents: HANLEY, Elizabeth, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US). | | | |

(54) Title: GENE THERAPY FOR CYSTIC FIBROSIS

(57) Abstract

Gene Therapy vectors, which are especially useful for cystic fibrosis, and methods for using the vectors are disclosed. In preferred embodiments, the vectors are adenovirus-based. Advantages of adenovirus-based vectors for gene therapy are that they appear to be relatively safe and can be manipulated to encode the desired gene product and at the same time are inactivated in terms of their ability to replicate in a normal lytic viral life cycle. Additionally, adenovirus has a natural tropism for airway epithelia. Therefore, adenovirus-based vectors are particularly preferred for respiratory gene therapy applications such as gene therapy for cystic fibrosis. In one embodiment, the adenovirus-based gene therapy vector comprises an adenovirus 2 serotype genome in which the E1a and E1b regions of the genome, which are involved in early stages of viral replication have been deleted and replaced by genetic material of interest (e.g., DNA encoding the cystic fibrosis transmembrane regulator protein). In another embodiment, the adenovirus-based therapy vector is a pseudo-adenovirus (PAV). PAVs contain no potentially harmful viral genes, have a theoretical capacity for foreign material of nearly 36 kb, may be produced in reasonably high titers and maintain the tropism of the parent adenovirus for dividing and non-dividing human target cell types.



72648-7

- 1 -

PSEUDO-ADENOVIRUS VECTORS

Background of the Invention

Cystic Fibrosis (CF) is the most common fatal genetic disease in humans (Boat, T.F. et al. in *The Metabolic Basis of Inherited Diseases* (Scriver, C.R. et al. eds., McGraw-Hill, New York (1989)). Approximately one in every 2,500 infants in the United States is born with the disease. At the present time, there are approximately 30,000 CF patients in the United States. Despite current standard therapy, the median age of survival is only 26 years. Disease of the pulmonary airways is the major cause of morbidity and is responsible for 95% of the mortality. The first manifestation of lung disease is often a cough, followed by progressive dyspnea. Tenacious sputum becomes purulent because of colonization of Staphylococcus and then with Pseudomonas. Chronic bronchitis and bronchiectasis can be partially treated with current therapy, but the course is punctuated by increasingly frequent exacerbations of the pulmonary disease. As the disease progresses, the patient's activity is progressively limited. End-stage lung disease is heralded by increasing hypoxemia, pulmonary hypertension, and cor pulmonale.

The upper airways of the nose and sinuses are also involved in CF. Most patients with CF develop chronic sinusitis. Nasal polyps occur in 15-20% of patients and are common by the second decade of life. Gastrointestinal problems are also frequent in CF; infants may suffer meconium ileus. Exocrine pancreatic insufficiency, which produces symptoms of malabsorption, is present in the large majority of patients with CF. Males are almost uniformly infertile and fertility is decreased in females.

Based on both genetic and molecular analyses, a gene associated with CF was isolated as part of 21 individual cDNA clones and its protein product predicted (Kerem, B.S. et al. (1989) *Science* 245:1073-1080; Riordan, J.R. et al. (1989) *Science* 245:1066-1073;

72648-7

-2-

Rommens, J.M. et al. (1989) *Science* 245:1059-1065)). (See also Gregory, R.J. et al. (1990) *Nature* 347:382-386; Rich, D.P. et al. (1990) *Nature* 347:358-362). The co-pending patent application also discloses experiments which show that proteins expressed from the wild type but not a mutant version of the cDNA complemented the defect in the cAMP regulated chloride channel shown previously to be characteristic of CF.

The protein product of the CF associated gene is called the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan, J.R. et al. (1989) *Science* 245:1066-1073). CFTR is a protein of approximately 1480 amino acids made up of two repeated elements, each comprising six transmembrane segments and a nucleotide binding domain. The two repeats are separated by a large, polar, so-called R-domain containing multiple potential phosphorylation sites. Based on its predicted domain structure, CFTR is a member of a class of related proteins which includes the multi-drug resistance (MDR) or P-glycoprotein, bovine adenylyl cyclase, the yeast STE6 protein as well as several bacterial amino acid transport proteins (Riordan, J.R. et al. (1989) *Science* 245:1066-1073; Hyde, S.C. et al. (1990) *Nature* 346:362-365). Proteins in this group, characteristically, are involved in pumping molecules into or out of cells.

CFTR has been postulated to regulate the outward flow of anions from epithelial cells in response to phosphorylation by cyclic AMP-dependent protein kinase or protein kinase C (Riordan, J.R. et al. (1989) *Science* 245:1066-1073; Welsh, 1986; Frizzell, R.A. et al. (1986) *Science* 233:558-560; Welsh, M.J. and Liedtke, C.M. (1986) *Nature* 322:467; Li, M. et al. (1988) *Nature* 331:358-360; Huang, T-C. et al. (1989) *Science* 244:1351-1353).

Sequence analysis of the CFTR gene of CF chromosomes has revealed a variety of mutations (Cutting, G.R. et al. (1990) *Nature* 346:366-369; Dean, M. et al. (1990) *Cell* 61:863-870; and Kerem, B-S. et al. (1989) *Science* 245:1073-1080; Kerem, B-S. et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:8447-8451). Population studies have indicated that the most common CF mutation, a deletion of the 3 nucleotides that encode phenylalanine at position 508 of the CFTR amino acid sequence ($\Delta F508$), is associated with approximately 70% of the cases of cystic fibrosis. This mutation results in the failure of an epithelial cell chloride channel to respond to cAMP (Frizzell R.A. et al. (1986) *Science* 233:558-560; Welsh, M.J. (1986) *Science* 232:1648-1650.; Li, M. et al. (1988) *Nature* 331:358-360; Quinton, P.M. (1989) *Clin. Chem.* 35:726-730). In airway cells, this leads to an imbalance in ion and fluid transport. It is widely believed that this causes abnormal mucus secretion, and ultimately results in pulmonary infection and epithelial cell damage.

Studies on the biosynthesis (Cheng, S.H. et al. (1990) *Cell* 63:827-834; Gregory, R.J. et al. (1991) *Mol. Cell Biol.* 11:3886-3893) and localization (Denning, G.M. et al. (1992) *J. Cell Biol.* 118:551-559) of CFTR $\Delta F508$, as well as other CFTR mutants, indicate that many CFTR mutant proteins are not processed correctly and, as a result, are not delivered to the

plasma membrane (Gregory, R.J. et al. (1991) *Mol. Cell Biol.* 11:3886-3893). These conclusions are consistent with earlier functional studies which failed to detect cAMP-stimulated Cl^- channels in cells expressing CFTR ΔF508 (Rich, D.P. et al. (1990) *Nature* 347:358-363; Anderson, M.P. et al. (1991) *Science* 251:679-682).

- 5 To date, the primary objectives of treatment for CF have been to control infection, promote mucus clearance, and improve nutrition (Boat, T.F. et al. in *The Metabolic Basis of Inherited Diseases* (Scriver, C.R. et al. eds., McGraw-Hill, New York (1989)). Intensive antibiotic use and a program of postural drainage with chest percussion are the mainstays of therapy. However, as the disease progresses, frequent hospitalizations are required.
- 10 Nutritional regimens include pancreatic enzymes and fat-soluble vitamins. Bronchodilators are used at times. Corticosteroids have been used to reduce inflammation, but they may produce significant adverse effects and their benefits are not certain. In extreme cases, lung transplantation is sometimes attempted (Marshall, S. et al. (1990) *Chest* 98:1488).

- Most efforts to develop new therapies for CF have focused on the pulmonary
- 15 complications. Because CF mucus consists of a high concentration of DNA, derived from lysed neutrophils, one approach has been to develop recombinant human DNase (Shak, S. et al. (1990) *Proc. Natl. Sci. Acad USA* 87:9188). Preliminary reports suggest that aerosolized enzyme may be effective in reducing the viscosity of mucus. This could be helpful in clearing the airways of obstruction and perhaps in reducing infections. In an attempt to limit
- 20 damage caused by an excess of neutrophil derived elastase, protease inhibitors have been tested. For example, alpha-1-antitrypsin purified from human plasma has been aerosolized to deliver enzyme activity to lungs of CF patients (McElvaney, N. et al. (1991) *The Lancet* 337:392). Another approach would be the use of agents to inhibit the action of oxidants derived from neutrophils. Although biochemical parameters have been successfully
- 25 measured, the long term beneficial effects of these treatments have not been established.

- Using a different rationale, other investigators have attempted to use pharmacological agents to reverse the abnormally decreased chloride secretion and increased sodium absorption in CF airways. Defective electrolyte transport by airway epithelia is thought to alter the composition of the respiratory secretions and mucus (Boat, T.F. et al. in *The*
- 30 *Metabolic Basis of Inherited Diseases* (Scriver, C.R. et al. eds., McGraw-Hill, New York (1989); Quinton, P.M. (1990) *FASEB J.* 4:2709-2717). Hence, pharmacological treatments aimed at correcting the abnormalities in electrolyte transport could be beneficial. Trials are in progress with aerosolized versions of the drug amiloride; amiloride is a diuretic that inhibits sodium channels, thereby inhibiting sodium absorption. Initial results indicate that the drug
- 35 is safe and suggest a slight change in the rate of disease progression, as measured by lung function tests (Knowles, M. et al. (1990) *N. Eng. J. Med.* 322: 1189-1194; App, E. (1990) *Am. Rev. Respir. Dis.* 141:605). Nucleotides, such as ATP or UTP, stimulate purinergic receptors in the airway epithelium. As a result, they open a class of chloride channel that is different from CFTR chloride channels. *In vitro* studies indicate that ATP and UTP can stimulate

2145641

- 4 -

chloride secretion (Knowles, M. et al. (1991) *N. Eng. J. Med.* 325:533). Preliminary trials to test the ability of nucleotides to stimulate secretion *in vivo*, and thereby correct the electrolyte transport abnormalities are underway.

5 Despite progress in therapy, cystic fibrosis remains a lethal disease, and no current therapy treats the basic defect. However, two general approaches may prove feasible. These are: 1) protein replacement therapy to deliver the wild type protein to patients to augment their defective protein, and; 2) gene replacement therapy to deliver wild type copies of the CF associated gene. Since the most life threatening manifestations of CF involve pulmonary complications, epithelial cells of the upper airways are appropriate target cells for therapy.

10 The feasibility of gene therapy has been established by introducing a wild type cDNA into epithelial cells from a CF patient and demonstrating complementation of the hallmark defect in chloride ion transport (Rich, D.P. et al. (1990) *Nature* 347:358-363). This initial work involved cells in tissue culture, however, subsequent work has shown that to deliver the gene to the airways of whole animals, defective adenoviruses may be useful (Rosenfeld, 15 (1992) *Cell* 68:143-155). However, the safety and effectiveness of using defective adenoviruses remain to be demonstrated.

Summary of the Invention

In general, the instant invention relates to vectors for transferring selected genetic material of interest (e.g., DNA or RNA) to cells *in vivo*. In preferred embodiments, the 20 vectors are adenovirus-based. Advantages of adenovirus-based vectors for gene therapy are that they appear to be relatively safe and can be manipulated to encode the desired gene product and at the same time are inactivated in terms of their ability to replicate in a normal lytic viral life cycle. Additionally, adenovirus has a natural tropism for airway epithelia. 25 Therefore, adenovirus-based vectors are particularly preferred for respiratory gene therapy applications such as gene therapy for cystic fibrosis.

In one embodiment, the adenovirus-based gene therapy vector comprises an adenovirus 2 serotype genome in which the Ela and Elb regions of the genome, which are involved in early stages of viral replication have been deleted and replaced by genetic 30 material of interest (e.g., DNA encoding the cystic fibrosis transmembrane regulator protein).

In another embodiment, the adenovirus-based therapy vector is a pseudo-adenovirus (PAV). PAVs contain no potentially harmful viral genes, have a theoretical capacity for foreign material of nearly 36 kb, may be produced in reasonably high titers and maintain the tropism of the parent adenovirus for dividing and non-dividing human target cell types. 35 PAVs comprise adenovirus inverted terminal repeats and the minimal sequences of a wild-type adenovirus type 2 genome necessary for efficient replication and packaging by a helper virus and genetic material of interest. In a preferred embodiment, the PAV contains adenovirus 2 sequences.

72648-7

5

In a further embodiment, the adenovirus-based gene therapy vector contains the open reading frame 6 (ORF6) of adenoviral early region 4 (E4) from the E4 promoter and is deleted for all other E4 open reading frames. Optionally, this vector can include deletions in the E1 and/or E3 regions. Alternatively, the adenovirus-based gene therapy vector contains the open reading frame 3 (ORF3) of adenoviral E4 from the E-4 promoter and is deleted for all other E4 open reading frames. Again, optionally, this vector can include deletions in the E1 and/or E3 regions. The deletion of non-essential open reading frames of E4 increases the cloning capacity by approximately 2 kb without significantly reducing the viability of the virus in cell culture. In combination with deletions in the E1 and/or E3 regions of adenovirus vectors, the theoretical insert capacity of the resultant vectors is increased to 8-9 kb.

The invention also relates to methods of gene therapy using the disclosed vectors and genetically engineered cells produced by the method.

One aspect of the invention provides an adenovirus-based gene therapy vector comprising the genome of an adenovirus 2 serotype in which the E1a and E1b regions of the genome, which are involved in early stages of viral replication, have been deleted.

Another aspect of the invention provides an adenovirus-based gene therapy vector comprising adenovirus inverted terminal repeat nucleotide sequences and nucleotide sequences necessary for efficient replication and packaging of genetic material of interest.

Another aspect of the invention provides an adenovirus-based gene therapy vector comprising an adenovirus

72648-7

5a

genome in which all E4 open reading frames except open reading frame 6 have been deleted.

Another aspect of the invention provides an adenovirus-based gene therapy vector comprising an adenovirus
5 genome in which all E4 open reading frames except open reading frame 3 have been deleted.

Another aspect of the invention provides use of a gene therapy vector comprising DNA encoding cystic fibrosis transmembrane conductance regulator, for treating or preventing
10 cystic fibrosis.

Another aspect of the invention provides use of a gene therapy vector comprising DNA encoding CFTR for the manufacture of a medicament for treating or preventing cystic fibrosis.

15 Another aspect of the invention provides a system for treating or preventing cystic fibrosis in a primate, comprising: a) a gene therapy vector comprising DNA encoding CFTR; and, b) means for delivering the vector to primate pulmonary airways.

20 Another aspect of the invention provides an adenoviral vector comprising a DNA sequence encoding a cystic fibrosis regulator transmembrane (CFTR) protein, wherein the DNA sequence comprises one or more alterations in order to eliminate one or more cryptic regulatory signals, wherein the
25 alteration in DNA sequence does not alter the amino acid sequence of the protein thereby encoded.

Another aspect of the invention provides an adenoviral vector comprising a DNA sequence encoding a cystic fibrosis regulator transmembrane (CFTR) protein, wherein the

72648-7

5b

DNA sequence has been altered to include a synthetic intron which is removed upon expression in a eukaryotic cell.

Another aspect of this invention provides an adenoviral vector comprising an adenovirus genome from which the E1, E2, E3, and E4 regions and late genes of the adenovirus genome have been deleted, a genetic material of interest operably linked to expression control sequences and the 5' and 3' inverted terminal repeat sequences, 5' packaging sequences and the E1A enhancer sequences of the adenovirus genome.

10 Another aspect of this invention provides a pharmaceutical composition comprising the adenoviral vector according to the invention and a suitable carrier or diluent.

Another aspect of this invention provides a pseudo-adenovirus (PAV) I vector.

15 Another aspect of this invention provides a pseudo-adenovirus (PAV) II vector.

Another aspect of this invention provides use of the adenoviral vector of the invention, wherein the genetic material of interest comprises DNA encoding cystic fibrosis transmembrane conductance regulator, for treating or preventing cystic fibrosis, and for the manufacture of a medicament therefor.

Another aspect of this invention provides a commercial package comprising the adenoviral vector according to the invention together with instructions for use for treating or preventing cystic fibrosis. In a preferred embodiment, the commercial package comprises an adenoviral vector of the invention comprising DNA encoding cystic fibrosis transmembrane conductance regulator.

72648-7

5c

Another aspect of this invention provides a system for treating or preventing cystic fibrosis in a primate, comprising: a) an adenoviral vector comprising DNA encoding cystic fibrosis transmembrane conductance regulator; and, b) means for delivering the vector to primate pulmonary airways, wherein the adenoviral vector is according to the invention and wherein the genetic material of interest comprises DNA encoding cystic fibrosis transmembrane conductance regulator.

Brief Description of the Tables and Drawings

Further understanding of the invention may be had by reference to the tables and figures wherein:

Table I shows CFTR mutants wherein the known association with CF (Y, yes or N, no), exon localization, domain location and presence (+) or absence (-) of bands A, B, and C of mutant CFTR species is shown. TM6, indicates transmembrane domain 6; NBD nucleotide binding domain; ECD, extracellular domain and Term, termination at 21 codons past residue 1337;

Table II shows the nucleotide sequence of Ad2/CFTR-1;

Table III depicts a nucleotide analysis of Ad2-ORF6/PGK-CFTR;

The convention for naming mutants is first the amino acid normally found at the particular residue, the residue number (Riordan, T.R. et al. (1989) *Science* 245:1066-1073), and the amino acid to which the residue was converted. The single letter amino acid code is used: D, aspartic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; W, tryptophan. Thus G551D is a mutant in which glycine 551 is converted to aspartic acid;

72648-7

5d

Figure 1 shows alignment of CFTR partial cDNA clones used in construction of cDNA containing complete coding sequence of the CFTR, only restriction sites relevant to the DNA constructions described below are shown;

5 Figure 2 depicts plasmid construction of the CFTR cDNA clone pKK-CFTR1;

2145641

- 6 -

Figure 3 depicts plasmid construction of the CFTR cDNA clone pKK-CFTR2;

Figure 4 depicts plasmid construction of the CFTR cDNA clone pSC-CFTR2;

5

Figure 5 shows a plasmid map of the CFTR cDNA clone pSC-CFTR2;

Figure 6 shows the DNA sequence of synthetic DNAs used for insertion of an intron into the CFTR cDNA sequence, with the relevant restriction endonuclease sites and nucleotide positions noted;

10

Figures 7A and 7B depict plasmid construction of the CFTR cDNA clone pKK-CFTR3;

Figure 8 shows a plasmid map of the CFTR cDNA pKK-CFTR3 containing an intron between nucleotides 1716 and 1717;

15

Figure 9 shows treatment of CFTR with glycosidases;

Figures 10A and 10B show an analysis of CFTR expressed from COS-7 transfected cells;

20

Figures 11A and 11B show pulse-chase labeling of wild type and $\Delta F508$ mutant CFTR in COS-7 transfected cells;

25

Figures 12A-12D show immunolocalization of wild type and $\Delta F508$ mutant CFTR; and COS-7 cells transfected with pMT-CFTR or pMT-CFTR- $\Delta F508$;

Figure 13 shows an analysis of mutant forms of CFTR;

30

Figure 14 shows a map of the first generation adenovirus based vector encoding CFTR (Ad2/CFTR-1);

Figure 15 shows the plasmid construction of the Ad2/CFTR-1 vector;

35

Figure 16 shows an example of UV fluorescence from an agarose gel electrophoresis of products of nested RT-PCR from lung homogenates of cotton rats which received Ad2/CFTR-1. The gel demonstrates that the homogenates were positive for virally-encoded CFTR mRNA;

Figure 17 shows an example of UV fluorescence from an agarose gel electrophoresis of products of nested RT-PCR from organ homogenates of cotton rats. The gel demonstrates that all organs of the infected rats were negative for Ad2/CFTR with the exception of the small bowel;

Figures 18A and 18B show differential cell analyses of bronchoalveolar lavage specimens from control and infected rats. These data demonstrate that none of the rats treated with Ad2/CFTR-1 had a change in the total or differential white blood cell count 4, 10, and 14 days after infection (Figure 18A) and 3, 7, and 14 days after infection (Figure 18B);

Figure 19 shows hematoxilyn and eosin stained sections of cotton rat tracheas from both treated and control rats sacrificed at different time points after infection with Ad2/CFTR-1. The sections demonstrate that there were no observable differences between the treated and control rats;

Figures 20A and 20B show examples of UV fluorescence from an agarose gel electrophoresis, stained with ethidium bromide, of products of RT-PCR from nasal brushings of Rhesus monkeys after application of Ad2/CFTR-1 or Ad2/ β -Gal;

Figure 21 shows lights microscopy and immunocytochemistry from monkey nasal brushings. The microscopy revealed that there was a positive reaction when nasal epithelial cells from monkeys exposed to Ad2/CFTR-1 were stained with antibodies to CFTR;

Figure 22 shows immunocytochemistry of monkey nasal turbinate biopsies. This microscopy reveals increased immunofluorescence at the apical membrane of the surface epithelium from biopsies obtained from monkeys treated with Ad2/CFTR-1 over that seen at the apical membrane of the surface epithelium from biopsies obtained from control monkeys;

Figures 23A-23D show serum antibody titers in Rhesus monkeys after three vector administrations. These graphs demonstrate that all three monkeys treated with Ad2/CFTR-1 developed antibodies against adenovirus;

Figure 24 shows hematoxilyn and eosin stained sections from monkey medial turbinate biopsies. These sections demonstrate that turbinate biopsy specimens from control monkeys could not be differentiated from those from monkeys treated with Ad2/CFTR-1 when reviewed by an independent pathologist;

2145641

Figures 25A-25I show photomicrographs of human nasal mucosa immediately before, during, and after Ad2/CFTR-1 application. These photomicrographs demonstrate that inspection of the nasal mucosa showed mild to moderate erythema, edema, and exudate in patients treated with Ad2/CFTR-1 (Figures 25A-25C) and in control patients (Figures 25G-25I). These changes were probably due to local anesthesia and vasoconstriction because when an additional patient was exposed to Ad2/CFTR in a method which did not require the use of local anesthesia or vasoconstriction, there were no symptoms and the nasal mucosa appeared normal (Figures 25D-25F);

Figure 26 shows a photomicrograph of a hematoxylin and eosin stained biopsy of human nasal mucosa obtained from the third patient three days after Ad2/CFTR-1 administration. This section shows a morphology consistent with CF, i.e., a thickened basement membrane and occasional morphonuclear cells in the submucosa, but no abnormalities that could be attributed to the adenovirus vector;

Figure 27 shows transepithelial voltage (V_t) across the nasal epithelium of a normal human subject. Amiloride (μM) and terbutaline (μM) were perfused onto the mucosal surface beginning at the times indicated. Under basal conditions (V_t) was electrically negative. Perfusion of amiloride onto the mucosal surface inhibited (V_t) by blocking apical Na^+ channels;

Figures 28A and 28B show transepithelial voltage (V_t) across the nasal epithelium of normal human subjects (Figure 28A) and patients with CF (Figure 28B). Values were obtained under basal conditions, during perfusion with amiloride (μM), and during perfusion of amiloride plus terbutaline (μM) onto the mucosal surface. Data are from seven normal subjects and nine patients with CF. In patients with CF, (V_t) was more electrically negative than in normal subjects (Figure 28B). Amiloride inhibited (V_t) in CF patients, as it did in normal subjects. However, V_t failed to hyperpolarize when terbutaline was perfused onto the epithelium in the presence of amiloride. Instead, (V_t) either did not change or became less negative, a result very different from that observed in normal subjects;

Figures 29A and 29B show transepithelial voltage (V_t) across the nasal epithelium of a third patient before (Figure 29A) and after (Figure 29B) administration of approximately 25 MOI of Ad2/CFTR-1. Amiloride and terbutaline were perfused onto the mucosal surface beginning at the times indicated. Figure 29A shows an example from the third patient before treatment. Figure 29B shows that in contrast to the response before Ad2/CFTR-1 was applied, after virus replication, in the presence of amiloride, terbutaline stimulated V_t ;

Figures 30A-30F show the time of course changes in transepithelial electrical properties before and after administration of Ad2/CFTR-1. Figures 30A and 30B are from the first patient who received approximately 1 MOI; Figures 30C and 30D are from the second patient who received approximately 3 MOI; and Figures 30E and 30F are from the third patient who received approximately 25 MOI. Figures 30A, 30C, and 30E show values of basal transepithelial voltage (V_t) and Figures 30B, 30D, and 30F show the change in transepithelial voltage (ΔV_t) following perfusion of terbutaline in the presence of amiloride. Day zero indicates the day of Ad2/CFTR-1 administration. Figures 30A, 30C, and 30E show the time course of changes in basal V_t for all three patients. The decrease in basal V_t suggests that application of Ad2/CFTR-1 corrected the CF electrolyte transport defect in nasal epithelium of all three patients. Additional evidence came from an examination of the response to terbutaline. Figures 30B, 30D, and 30F show the time course of the response. These data indicate that Ad2/CFTR-1 corrected the CF defect in Cl^- transport;

Figure 31 shows the time course of changes in transepithelial electrical properties before and after administration of saline instead of Ad2/CFTR-1 to CF patients. Day zero indicates the time of mock administration. The top graph shows basal transepithelial voltage (V_t) and the bottom graph shows the change in transepithelial voltage following perfusion with terbutaline in the presence of amiloride (ΔV_t). Closed symbols are data from two patients that received local anesthetic/vasoconstriction and placement of the applicator for thirty minutes. Open symbol is data from a patient that received local anesthetic/vasoconstriction, but not placement of the applicator. Symptomatic changes and physical findings were the same as those observed in CF patients treated with a similar administration procedure and Ad2/CFTR-1;

Figure 32 shows a map of the second generation adenovirus based vector, PAV;

Figure 33 shows the plasmid construction of a second generation adenoviral vector 6 (Ad E4 ORF6);

Figure 34 is a schematic of Ad2-ORF6/PGK-CFTR which differs from Ad2/CFTR in that the latter utilized the endogenous Ela promoter, had no poly A addition signal directly downstream of CFTR and retained an intact E4 region;

Figure 35 shows short-circuit currents from human CF nasal polyp epithelial cells infected with Ad2-ORF6/PGK-CFTR at multiplicities of 0.3, 3, and 50. At the indicated times: (1) 10 μ M amiloride, (2) cAMP agonists (10 μ M forskolin and 100 μ M IBMX, and (3) 1 mM diphenylamine-2-carboxylate were added to the mucosal solution;

2145641

- 10 -

Figures 36A-36D show immunocytochemistry of nasal brushings by laser scanning microscopy of the Rhesus monkey C, before infection (36A) and on 7 days (36B); 24 (36C); and 38 (36D) after the first infection with Ad2-ORF6/PGK-CFTR;

Figures 37A-37D show immunocytochemistry of nasal brushings by laser scanning microscopy of Rhesus monkey D, before infection (37A) and on days 7 (37B); 24 (37C); and 48 (37D) after the first infection with Ad2-ORF6/PGK-CFTR;

Figures 38A-38D show immunocytochemistry of nasal brushings by laser scanning microscopy of the Rhesus monkey E, before infection (38A) and on days 7 (38B); 24 (38C); and 48 (38D) after the first infection with Ad2-ORF6/PGK-CFTR;

Figures 39A-39C show summaries of the clinical signs (or lack thereof) of infection with Ad2-ORF6/PGK-CFTR;

Figures 40A-40C shows a summary of blood counts, sedimentation rate, and clinical chemistries after infection with Ad2-ORF6/PGK-CFTR for monkeys C, D, and E. There was no evidence of a systemic inflammatory response or other abnormalities of the clinical chemistries;

Figure 41 shows summaries of white blood cells counts in monkeys C, D, and E after infection with Ad2-ORF6/PGK-CFTR. These data indicate that the administration of Ad2-ORF6/PGK-CFTR caused no change in the distribution and number of inflammatory cells at any of the time points following viral administration;

Figure 42 shows histology of submucosal biopsy performed on Rhesus monkey C on day 4 after the second viral instillation of Ad2-ORF6/PGK-CFTR. Hematoxylin and eosin stain revealed no evidence of inflammation or cytopathic changes;

Figure 43 shows histology of submucosal biopsy performed on Rhesus monkey D on day 11 after the second viral instillation of Ad2-ORF6/PGK-CFTR. Hematoxylin and eosin stain revealed no evidence of inflammation or cytopathic changes;

Figure 44 shows histology of submucosal biopsy performed on Rhesus monkey E on day 18 after the second viral instillation of Ad2-ORF6/PGK-CFTR. Hematoxylin and eosin stain revealed no evidence of inflammation or cytopathic changes; and

Figures 45A-45C show antibody titers to adenovirus prior to and after the first and second administrations of Ad2-ORF6/PGK-CFTR. Prior to administration of Ad2-ORF6/PGK-

2145641

CFTR, the monkeys had received instillations of Ad2/CFTR-1. Antibody titers measured by ELISA rose within one week after the first and second administrations of Ad2-ORF6/PGK-CFTR. Serum neutralizing antibodies also rose within a week after viral administration and peaked at day 24. No anti-adenoviral antibodies were detected by ELISA or neutralizing assay in nasal washings of any of the monkeys.

Detailed Description and Best Mode

Gene Therapy

As used herein, the phrase "gene therapy" refers to the transfer of genetic material (e.g., DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition. The genetic material of interest encodes a product (e.g., a protein polypeptide, peptide or functional RNA) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme or (poly) peptide of therapeutic value. Examples of genetic material of interest include DNA encoding: the cystic fibrosis transmembrane regulator (CFTR), Factor VIII, low density lipoprotein receptor, beta-galactosidase, alpha-galactosidase, beta-glucocerebrosidase, insulin, parathyroid hormone, and alpha-1-antitrypsin.

Although the potential for gene therapy to treat genetic diseases has been appreciated for many years, it is only recently that such approaches have become practical with the treatment of two patients with adenosine deaminase deficiency. The protocol consists of removing lymphocytes from the patients, stimulating them to grow in tissue culture, infecting them with an appropriately engineered retrovirus followed by reintroduction of the cells into the patient (Kantoff, P. et al. (1987) *J. Exp. Med.* 166:219). Initial results of treatment are very encouraging. With the approval of a number of other human gene therapy protocols for limited clinical use, and with the demonstration of the feasibility of complementing the CF defect by gene transfer, gene therapy for CF appears a very viable option.

The concept of gene replacement therapy for cystic fibrosis is very simple; a preparation of CFTR coding sequences in some suitable vector in a viral or other carrier delivered directly to the airways of CF patients. Since disease of the pulmonary airways is the major cause of morbidity and is responsible for 95% of mortality, airway epithelial cells are preferred target cells for CF gene therapy. The first generation of CF gene therapy is likely to be transient and to require repeated delivery to the airways. Eventually, however, gene therapy may offer a cure for CF when the identity of the precursor or stem cell to air epithelial cells becomes known. If DNA were incorporated into airway stem cells, all subsequent generations of such cells would make authentic CFTR from the integrated sequences and would correct the physiological defect almost irrespective of the biochemical basis of the action of CFTR.

Although simple in concept, scientific and clinical problems face approaches to gene therapy, not least of these being that CF requires an *in vivo* approach while all gene therapy treatments in humans to date have involved *ex vivo* treatment of cells taken from the patient followed by reintroduction.

- 5 One major obstacle to be overcome before gene therapy becomes a viable treatment approach for CF is the development of appropriate vectors to infect tissue manifesting the disease and deliver the therapeutic CFTR gene. Since viruses have evolved very efficient means to introduce their nucleic acid into cells, many approaches to gene therapy make use of engineered defective viruses. However, the use of viruses *in vivo* raises safety concerns.
- 10 Although potentially safer, the use of simple DNA plasmid constructs containing minimal additional DNA, on the other hand, is often very inefficient and can result in transient protein expression.

- The integration of introduced DNA into the host chromosome has advantages in that such DNA will be passed to daughter cells. In some circumstances, integrated DNA may
- 15 also lead to high or more sustained expression. However, integration often, perhaps always, requires cellular DNA replication in order to occur. This is certainly the case with the present generation of retroviruses. This limits the use of such viruses to circumstances where cell division occurs in a high proportion of cells. For cells cultured *in vitro*, this is seldom a problem, however, the cells of the airway are reported to divide only infrequently
- 20 (Kawanami, O. et al. (1979) *An. Rev. Respir. Dis.* 120:595). The use of retroviruses in CF will probably require damaging the airways (by agents such as SO₂ or O₃) to induce cell division. This may prove impracticable in CF patients.

- Even if efficient DNA integration could be achieved using viruses, the human genome contains elements involved in the regulation of cellular growth only a small fraction of which
- 25 are presently identified. By integrating adjacent to an element such as a proto-oncogene or an anti-oncogene, activation or inactivation of that element could occur leading to uncontrolled growth of the altered cell. It is considered likely that several such activation/inactivation steps are usually required in any one cell to induce uncontrolled proliferation (R.A. Weinberg (1989) *Cancer Research* 49:3713), which may reduce somewhat the potential risk. On the
- 30 other hand, insertional mutagenesis leading to tumor formation is certainly known in animals with some nondefective retroviruses (R.A. Weinberg, *supra*; Payne, G.S. et al. (1982) *Nature* 295:209), and the large numbers of potential integrations occurring during the lifetime of a patient treated repeatedly *in vivo* with retroviruses must raise concerns on the safety of such a procedure.

- 35 In addition to the potential problems associated with viral DNA integration, a number of additional safety issues arise. Many patients may have preexisting antibodies to some of the viruses that are candidates for vectors, for example, adenoviruses. In addition, repeated use of such vectors might induce an immune response. The use of defective viral vectors

2145641

- 13 -

may alleviate this problem somewhat, because the vectors will not lead to productive viral life cycles generating infected cells, cell lysis or large numbers of progeny viruses.

Other issues associated with the use of viruses are the possibility of recombination with related viruses naturally infecting the treated patient, complementation of the viral defects by simultaneous expression of wild type virus proteins and containment of aerosols of the engineered viruses.

Gene therapy approaches to CF will face many of the same clinical challenges at protein therapy. These include the inaccessibility of airway epithelium caused by mucus build-up and the hostile nature of the environment in CF airways which may inactivate viruses/vectors. Elements of the vector carriers may be immunogenic and introduction of the DNA may be inefficient. These problems, as with protein therapy, are exacerbated by the absence of a good animal model for the disease nor a simple clinical end point to measure the efficacy of treatment.

15 CF Gene Therapy Vectors - Possible Options

Retroviruses - Although defective retroviruses are the best characterized system and so far the only one approved for use in human gene therapy (Miller, A.D. (1990) *Blood* 76:271), the major issue in relation to CF is the requirement for dividing cells to achieve DNA integration and gene expression. Were conditions found to induce airway cell division, the *in vivo* application of retroviruses, especially if repeated over many years, would necessitate assessment of the safety aspects of insertional mutagenesis in this context.

Adeno-Associated Virus - (AAV) is a naturally occurring defective virus that requires other viruses such as adenoviruses or herpes viruses as helper viruses (Muzyczka, N. (1992) in *Current Topics in Microbiology and Immunology* 158:97). It is also one of the few viruses that may integrate its DNA into non-dividing cells, although this is not yet certain. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. CFTR DNA may be towards the upper limit of packaging. Furthermore, the packaging process itself is presently inefficient and safety issues such as immunogenicity, complementation and containment will also apply to AAV. Nevertheless, this system is sufficiently promising to warrant further study.

Plasmid DNA - Naked plasmid can be introduced into muscle cells by injection into the tissue. Expression can extend over many months but the number of positive cells is low (Wolff, J. et al. (1989) *Science* 247:1465). Cationic lipids aid introduction of DNA into some cells in culture (Felgner, P. and Ringold, G.M. (1989) *Nature* 337:387). Injection of cationic lipid plasmid DNA complexes into the circulation of mice has been shown to result in expression of the DNA in lung (Brigham, K. et al. (1989) *Am. J. Med. Sci.* 298:278).

Instillation of cationic lipid plasmid DNA into lung also leads to expression in epithelial cells but the efficiency of expression is relatively low and transient (Hazinski, T.A. et al. (1991) *Am. J. Respir., Cell Mol. Biol.* 4:206). One advantage of the use of plasmid DNA is that it can be introduced into non-replicating cells. However, the use of plasmid DNA in the CF
5 airway environment, which already contains high concentrations of endogenous DNA may be problematic.

Receptor Mediated Entry - In an effort to improve the efficiency of plasmid DNA uptake, attempts have been made to utilize receptor-mediated endocytosis as an entry
10 mechanisms and to protect DNA in complexes with polylysine (Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621). One potential problem with this approach is that the incoming plasmid DNA enters the pathway leading from endosome to lysosome, where much incoming material is degraded. One solution to this problem is the use of transferrin DNA-polylysine complexes linked to adenovirus capsids (Curiel, D.T. et al. (1991) *Proc. Natl. Acad. Sci. USA*
15 88:8850). The latter enter efficiently but have the added advantage of naturally disrupting the endosome thereby avoiding shuttling to the lysosome. This approach has promise but at present is relatively transient and suffers from the same potential problems of immunogenicity as other adenovirus based methods.

Adenovirus - Defective adenoviruses at present appear to be a promising approach to CF gene therapy (Berkner, K.L. (1988) *BioTechniques* 6:616). Adenovirus can be manipulated such that it encodes and expresses the desired gene product, (e.g., CFTR), and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. In addition, adenovirus has a natural tropism for airway epithelia. The viruses are able to
25 infect quiescent cells as are found in the airways, offering a major advantage over retroviruses. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about insertional mutagenesis. Furthermore, adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile (Schwartz, A.R. et al. (1974) *Am. Rev. Respir. Dis.* 109:233-238).
30 Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances including transfer of alpha-1-antitrypsin and CFTR to the lungs of cotton rats (Rosenfeld, M.A. et al. (1991) *Science* 252:431-434; Rosenfeld et al., (1992) *Cell* 68:143-155). Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative (Green, M. et al. (1979) *Proc. Natl. Acad. Sci. USA*
35 76:6606).

The following properties would be desirable in the design of an adenovirus vector to transfer the gene for CFTR to the airway cells of a CF patient. The vector should allow sufficient expression of the CFTR, while producing minimal viral gene expression. There should be minimal viral DNA replication and ideally no virus replication. Finally,

2145641

recombination to produce new viral sequences and complementation to allow growth of the defective virus in the patient should be minimized. A first generation adenovirus vector encoding CFTR (Ad2/CFTR), made as described in the following Example 7, achieves most of these goals and was used in the human trials described in Example 10.

5 Figure 14 shows a map of Ad2/CFTR-1. As can be seen from the figure, this first generation virus includes viral DNA derived from the common relatively benign adenovirus 2 serotype. The Ela and Elb regions of the viral genome, which are involved in early stages of viral replication have been deleted. Their removal impairs viral gene expression and viral replication. The protein products of these genes also have immortalizing and transforming
10 function in some non-permissive cells.

 The CFTR coding sequence is inserted into the viral genome in place of the Ela/Elb region and transcription of the CFTR sequence is driven by the endogenous Ela promoter. This is a moderately strong promoter that is functional in a variety of cells. In contrast to some adenovirus vectors (Rosenfeld, M. et al. (1992) *Cell* 68:143), this adenovirus retains
15 the E3 viral coding region. As a consequence of the inclusion of E3, the length of the adenovirus-CFTR DNA is greater than that of the wild-type adenovirus. The greater length of the recombinant viral DNA renders it more difficult to package. This means that the growth of the Ad2/CFTR virus is impaired even in permissive cells that provide the missing Ela and Elb functions.

20 The E3 region of the Ad2/CFTR-1 encodes a variety of proteins. One of these proteins, gp19, is believed to interact with and prevent presentation of class I proteins of the major histocompatibility complex (MHC) (Gooding, C.R. and Wold, W.S.M. (1990) *Crit. Rev. Immunol.* 10:53). This property prevents recognition of the infected cells and thus may allow viral latency. The presence of E3 sequences, therefore, has two useful attributes; first,
25 the large size of the viral DNA renders it doubly defective for replication (i.e., it lacks early functions and is packaged poorly) and second, the absence of MHC presentation could be useful in later applications of Ad2/CFTR-1 in gene therapy involving multiple administrations because it may avoid an immune response to recombinant virus containing cells.

30 Not only are there advantages associated with the presence of E3; there may be disadvantages associated with its absence. Studies of E3 deleted virus in animals have suggested that they result in a more severe pathology (Gingsberg, H.S. et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:3823). Furthermore, E3 deleted virus, such as might be obtained by recombination of an E1 plus E3 deleted virus with wild-type virus, is reported to outgrow
35 wild-type in tissue culture (Barkner, K.L. and Sharp, P. (1983) *Nucleic Acids Research* 11:6003). By contrast, however, a recent report of an E3 replacement vector encoding hepatitis B surface antigen, suggests that when delivered as a live enteric vaccine, such a virus replicates poorly in human compared to wild-type.

The adenovirus vector (Ad2/CFTR-1) and a related virus encoding the marker β -galactosidase (Ad2/ β -gal) have been constructed and grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express Ela and Elb, which complement the defective adenoviruses by providing the products of the genes deleted from the vector. Because the size of its genome is greater than that of wild-type virus, Ad2/CFTR is relatively difficult to produce.

The Ad2/CFTR-1 virus has been shown to encode CFTR by demonstrating the presence of the protein in 293 cells. The Ad2/ β -gal virus was shown to produce its protein in a variety of cell lines grown in tissue culture including a monkey bronchiolar cell line (4MBR-5), primary hamster tracheal epithelial cells, human HeLa, human CF PAC cells (see Example 8) and airway epithelial cells from CF patients (Rich, O. et al. (1990) *Nature* 347:358).

Ad2/CFTR-1 is constructed from adenovirus 2 (Ad2) DNA sequences. Other varieties of adenovirus (e.g., Ad3, Ad5, and Ad7) may also prove useful as gene therapy vectors. This may prove essential if immune response against a single serotype reduces the effectiveness of the therapy.

Second Generation Adenoviral Vectors

Adenoviral vectors currently in use retain most ($\geq 80\%$) of the parental viral genetic material leaving their safety untested and in doubt. Second-generation vector systems containing minimal adenoviral regulatory, packaging and replication sequences have therefore been developed.

Pseudo-Adenovirus Vectors (PAV)-PAVs contain adenovirus inverted terminal repeats and the minimal adenovirus 5' sequences required for helper virus dependent replication and packaging of the vector. These vectors contain no potentially harmful viral genes, have a theoretical capacity for foreign material of nearly 36 kb, may be produced in reasonably high titers and maintain the tropism of the parent virus for dividing and non-dividing human target cell types.

The PAV vector can be maintained as either a plasmid-borne construct or as an infectious viral particle. As a plasmid construct, PAV is composed of the minimal sequences from wild type adenovirus type 2 necessary for efficient replication and packaging of these sequences and any desired additional exogenous genetic material, by either a wild-type or defective helper virus.

Specifically, PAV contains adenovirus 2 (Ad2) sequences as shown in Figure 17, from nucleotide (nt) 0-356 forming the 5' end of the vector and the last 109 nt of Ad2 forming the 3' end of the construct. The sequences includes the Ad2 flanking inverted terminal repeats (5'ITR) and the 5' ITR adjoining sequences containing the known packaging signal and Ela enhancer. Various convenient restriction sites have been incorporated into the

2145641

- 17 -

fragments, allowing the insertion of promoter/gene cassettes which can be packaged in the PAV virion and used for gene transfer (e.g. for gene therapy). The construction and propagation of PAV is described in detail in the following Example 11. By not containing most native adenoviral DNA, the PAVs described herein are less likely to produce a patient immune response or to replicate in a host.

In addition, the PAV vectors can accommodate foreign DNA up to a maximum length of nearly 36 kb. The PAV vectors therefore, are especially useful for cloning larger genes (e.g., CFTR (7.5 kb)); Factor VIII (8 kb); Factor IX (9 kb)), which, traditional vectors have difficulty accommodating. In addition, PAV vectors can be used to transfer more than one gene, or more than one copy of a particular gene. For example, for gene therapy of cystic fibrosis, PAVs can be used to deliver CFTR in conjunction with other genes such as anti proteases (e.g., antiprotease alpha-1-antitrypsin) tissue inhibitor of metalloproteinase, antioxidants (e.g., superoxide dismutase), enhancers of local host defense (e.g., interferons), mucolytics (e.g., DNase); and proteins which block inflammatory cytokines.

Ad2-E4/ORF6 Adenovirus Vectors

An adenoviral construct expressing only the open reading frame 6 (ORF6) of adenoviral early region 4 (E4) from the E4 promoter and which is deleted for all other known E4 open reading frames was constructed as described in detail in Example 12. Expression of E4 open reading frame 3 is also sufficient to provide E4 functions required for DNA replication and late protein synthesis. However, it provides these functions with reduced efficiency compared to expression of ORF6, which will likely result in lower levels of virus production. Therefore expressing ORF6, rather than ORF3, appears to be a better choice for producing recombinant adenovirus vectors.

The E4 region of adenovirus is suspected to have a role in viral DNA replication, late mRNA synthesis and host protein synthesis shut off, as well as in viral assembly (Falgout, B. and G. Ketner (1987) *J. Virol.* 61:3759-3768). Adenovirus early region 4 is required for efficient virus particle assembly. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. Halbert, D.N. et al. (1985) *J. Virol.* 56:250-257.

The deletion of non-essential open reading frames of E4 increases the cloning capacity of recombinant adenovirus vectors by approximately 2 kb of insert DNA without significantly reducing the viability of the virus in cell culture. When placed in combination with deletions in the E1 and/or E3 regions of adenovirus vectors, the theoretical insert capacity of the resultant vectors is increased to 8-9 kb. An example of where this increased cloning capacity may prove useful is in the development of a gene therapy vector encoding CFTR. As described above, the first generation adenoviral vector approaches the maximum packaging capacity for viral DNA encapsidation. As a result, this virus grows poorly and may occasionally give rise to defective progeny. Including an E4 deletion in the adenovirus

vector should alleviate these problems. In addition, it allows flexibility in the choice of promoters to drive CFTR expression from the virus. For example, strong promoters such as the adenovirus major late promoter, the cytomegalovirus immediate early promoter or a cellular promoter such as the CFTR promoter, which may be too large for first-generation
5 adenovirus can be used to drive expression.

In addition, by expressing only ORF6 of E4, these second generation adenoviral vectors may be safer for use in gene therapy. Although ORF6 expression is sufficient for viral DNA replication and late protein synthesis in immortalized cells, it has been suggested that ORF6/7 of E4 may also be required in non-dividing primary cells (Hemstrom, C. et al.
10 (1991) *J. Virol.* 65:1440-1449). The 19 kD protein produced from open reading frame 6 and 7 (ORF6/7) complexes with and activates cellular transcription factor E2F, which is required for maximal activation of early region 2. Early region 2 encodes proteins required for viral DNA replication. Activated transcription factor E2F is present in proliferating cells and is involved in the expression of genes required for cell proliferation (e.g., DHFR, c-myc),
15 whereas activated E2F is present in lower levels in non-proliferating cells. Therefore, the expression of only ORF6 of E4 should allow the virus to replicate normally in tissue culture cells (e.g., 293 cells), but the absence of ORF6/7 would prevent the potential activation of transcription factor E2F in non-dividing primary cells and thereby reduce the potential for viral DNA replication.

20 Target Tissue

Because 95% of CF patients die of lung disease, the lung is a preferred target for gene therapy. The hallmark abnormality of the disease is defective electrolyte transport by the epithelial cells that line the airways. Numerous investigators (reviewed in Quinton, F. (1990)
25 *FASEB J.* 4:2709) have observed: a) a complete loss of cAMP-mediated transepithelial chloride secretion, and b) a two to three fold increase in the rate of Na⁺ absorption. cAMP-stimulated chloride secretion requires a chloride channel in the apical membrane (Welsh, M.J. (1987) *Physiol Rev.* 67:1143-1184). The discovery that CFTR is a phosphorylation-regulated chloride channel and that the properties of the CFTR chloride channel are the same as those
30 of the chloride channels in the apical membrane, indicate that CFTR itself mediates transepithelial chloride secretion. This conclusion was supported by studies localizing CFTR in lung tissue: CFTR is located in the apical membrane of airway epithelial cells (Denning, G.M. et al. (1992) *J. Cell Biol.* 118:551) and has been reported to be present in the submucosal glands (Taussig et al., (1973) *J. Clin. Invest.* 89:339). As a consequence of loss
35 of CFTR function, there is a loss of cAMP-regulated transepithelial chloride secretion. At this time it is uncertain how dysfunction of CFTR produces an increase in the rate of Na⁺ absorption. However, it is thought that the defective chloride secretion and increased Na⁺ absorption lead to an alteration of the respiratory tract fluid and hence, to defective mucociliary clearance, a normal pulmonary defense mechanism. As a result, clearance of

inhaled material from the lung is impaired and repeated infections ensue. Although the presumed abnormalities in respiratory tract fluid and mucociliary clearance provide a plausible explanation for the disease, a precise understanding of the pathogenesis is still lacking.

5 Correction of the genetic defect in the airway epithelial cells is likely to reverse the CF pulmonary phenotype. The identity of the specific cells in the airway epithelium that express CFTR cannot be accurately determined by immunocytochemical means, because of the low abundance of protein. However, functional studies suggest that the ciliated epithelial cells and perhaps nonciliated cells of the surface epithelium are among the main cell types
10 involved in electrolyte transport. Thus, in practical terms, the present preferred target cell for gene therapy would appear to be the mature cells that line the pulmonary airways. These are not rapidly dividing cells; rather, most of them are nonproliferating and many may be terminally differentiated. The identification of the progenitor cells in the airway is uncertain. Although CFTR may also be present in submucosal glands (Trezise, A.E. and Buchwald, M.
15 (1991) *Nature* 353:434; Englehardt, J.F. et al. (1992) *J. Clin. Invest.* 90:2598-2607), there is no data as to its function at that site; furthermore, such glands appear to be relatively inaccessible.

 The airway epithelium provides two main advantages for gene therapy. First, access to the airway epithelium can be relatively noninvasive. This is a significant advantage in the
20 development of delivery strategies and it will allow investigators to monitor the therapeutic response. Second, the epithelium forms a barrier between the airway lumen and the interstitium. Thus, application of the vector to the lumen will allow access to the target cell yet, at least to some extent, limit movement through the epithelial barrier to the interstitium and from there to the rest of the body.

25

Efficiency of Gene Delivery Required to Correct The Genetic Defect

 It is unlikely that any gene therapy protocol will correct 100% of the cells that normally express CFTR. However, several observations suggest that correction of a small
30 percent of the involved cells or expression of a fraction of the normal amount of CFTR may be of therapeutic benefit.

a. CF is an autosomal recessive disease and heterozygotes have no lung disease. Thus, 50% of wild-type CFTR would appear sufficient for normal function.

35 b. This issue was tested in mixing experiments using CF cells and recombinant CF cells expressing wild-type CFTR (Johnson, L.G. et al. (1992) *Nature Gen.* 2:21). The data obtained showed that when an epithelium is reconstituted with as few as 6-10% of corrected cells, chloride secretion is comparable to that observed with an epithelium containing 100% corrected cells. Although CFTR expression in the recombinant cells is

probably higher than in normal cells, this result suggests that *in vivo* correction of all CF airway cells may not be required.

5 c. Recent observations show that CFTR containing some CF-associated mutations retains residual chloride channel activity (Sheppard, D.N. et al. (1992) *Pediatr. Pulmon Suppl.* 8:250; Strong, T.V. et al. (1991) *N. Eng. J. Med.* 325:1630). These mutations are associated with mild lung disease. Thus, even a very low level of CFTR activity may at least partly ameliorate the electrolyte transport abnormalities.

10 d. As indicated in experiments described below in Example 8, complementation of CF epithelia, under conditions that probably would not cause expression of CFTR in every cell, restored cAMP stimulated chloride secretion.

15 e. Levels of CFTR in normal human airway epithelia are very low and are barely detectable. It has not been detected using routine biochemical techniques such as immunoprecipitation or immunoblotting and has been exceedingly difficult to detect with immunocytochemical techniques (Denning, G.M. et al. (1992) *J. Cell Biol.* 118:551). Although CFTR has been detected in some cases using laser-scanning confocal microscopy, the signal is at the limits of detection and cannot be detected above background in every case.
20 Despite that minimal levels of CFTR, this small amount is sufficient to generate substantial cAMP-stimulated chloride secretion. The reason that a very small number of CFTR chloride channels can support a large chloride secretory rate is that a large number of ions can pass through a single channel (10^6 - 10^7 ions/sec) (Hille, B. (1984) Sinauer Assoc. Inc., Sunderland, MA 420-426).

25 f. Previous studies using quantitative PCR have reported that the airway epithelial cells contain at most one to two transcripts per cell (Trapnell, B.C. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:6565).

30 Gene therapy for CF would appear to have a wide therapeutic index. Just as partial expression may be of therapeutic value, overexpression of wild-type CFTR appears unlikely to cause significant problems. This conclusion is based on both theoretical considerations and experimental results. Because CFTR is a regulated channel, and because it has a specific function in epithelia, it is unlikely that overexpression of CFTR will lead to uncontrolled
35 chloride secretion. First, secretion would require activation of CFTR by cAMP-dependent phosphorylation. Activation of this kinase is a highly regulated process. Second, even if CFTR chloride channels open in the apical membrane, secretion will not ensue without regulation of the basolateral membrane transporters that are required for chloride to enter the cell from the interstitial space. At the basolateral membrane, the sodium-potassium-chloride

72648-7

-21-

cotransporter and potassium channels serve as important regulators of transepithelial secretion (Welsh, M.J. (1987) *Physiol. Rev.* 67:1143-1184).

Human CFTR has been expressed in transgenic mice under the control of the surfactant protein C (SPC) gene promoter (Whitesett, J.A. et al. (1992) *Nature Gen.* 2:13) and the casein promoter (Ditullio, P. et al (1992) *Bio/Technology* 10:74). In those mice, CFTR was overexpressed in bronchiolar and alveolar epithelial cells and in the mammary glands, respectively. Yet despite the massive overexpression in the transgenic animals, there were no observable morphologic or functional abnormalities. In addition, expression of CFTR in the lungs of cotton rats produced no reported abnormalities (Rosenfeld, M.A. et al. (1992) *Cell* 68:143-155).

The present invention is further illustrated by the following examples which in no way should be construed as being further limiting.

EXAMPLES

Example 1 - Generation of Full Length CFTR cDNAs

Nearly all of the commonly used DNA cloning vectors are based on plasmids containing modified pMB1 replication origins and are present at up to 500 to 700 copies per cell (Sambrook et al. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press 1989). The partial CFTR cDNA clones isolated by Riordan et al. were maintained in such a plasmid. It was postulated that an alternative theory to intrinsic clone instability to explain the apparent inability to recover clones encoding full length CFTR protein using high copy number plasmids, was that it was not possible to clone large segments of the CFTR cDNA at high gene dosage in *E. coli*. Expression of the CFTR or portions of the CFTR from regulatory sequences capable of directing transcription and/or translation in the bacterial host cell might result in inviability of the host cell due to toxicity of the transcript or of the full length CFTR protein or fragments thereof. This inadvertent gene expression could occur from either plasmid regulatory sequences or cryptic regulatory sequences within the recombinant CFTR plasmid which are capable of functioning in *E. coli*. Toxic expression of the CFTR coding sequences would be greatly compounded if a large number of copies of the CFTR cDNA were present in cells because a high copy number plasmid was used. If the product was indeed toxic as postulated, the growth of cells containing full length and correct sequence would be actively disfavored. Based upon this novel hypothesis, the following procedures were undertaken. With reference to Figure 2, partial CFTR clone T16-4.5 was cleaved with restriction enzymes Sph I and Pst I and the resulting 3.9 kb restriction fragment containing exons 11 through most of exon 24 (including

an uncharacterized 119 bp insertion reported by Riordan et al. between nucleotides 1716 and 1717), was isolated by agarose gel purification and ligated between the Sph I and Pst I sites of the pMB1 based vector pkk223-3 (Brosius and Holy, (1984) *Proc. Natl. Acad. Sci.* 81:6929). It was hoped that the pMB1 origin contained within this plasmid would allow it and plasmids constructed from it to replicate at 15-20 copies per host *E. coli* cell (Sambrook et al. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press 1989). The resultant plasmid clone was called pkk-4.5.

Partial CFTR clone T11 was cleaved with Eco R I and Hinc II and the 1.9 kb band encoding the first 1786 nucleotides of the CFTR cDNA plus an additional 100 bp of DNA at the 5' end was isolated by agarose gel purification. This restriction fragment was inserted between the Eco R I site and Sma I restriction site of the plasmid Bluescript Sk- (Stratagene, catalogue number 212206), such that the CFTR sequences were now flanked on the upstream (5') side by a Sal I site from the cloning vector. This clone, designated T11-R, was cleaved with Sal I and Sph I and the resultant 1.8 kb band isolated by agarose gel purification.

Plasmid pkk-4.5 was cleaved with Sal I and Sph I and the large fragment was isolated by agarose gel purification. The purified T11-R fragment and pkk-4.5 fragments were ligated to construct pkk-CFTR1. pkk-CFTR1 contains exons 1 through 24 of the CFTR cDNA. It was discovered that this plasmid is stably maintained in *E. coli* cells and confers no measurably disadvantageous growth characteristics upon host cells.

pkk-CFTR1 contains, between nucleotides 1716 and 1717, the 119 bp insert DNA derived from partial cDNA clone T16-4.5 described above. In addition, subsequent sequence analysis of pkk-CFTR1 revealed unreported differences in the coding sequence between that portion of CFTR1 derived from partial cDNA clone T11 and the published CFTR cDNA sequence. These undesired differences included a 1 base-pair deletion at position 995 and a C to T transition at position 1507.

To complete construction of an intact correct CFTR coding sequence without mutations or insertions and with reference to the construction scheme shown in Figure 3, pkk-CFTR1 was cleaved with Xba I and Hpa I, and dephosphorylated with calf intestinal alkaline phosphatase. In addition, to reduce the likelihood of recovering the original clone, the small unwanted Xba I/Hpa I restriction fragment from pKK-CFTR1 was digested with Sph I. T16-1 was cleaved with Xba I and Acc I and the 1.15 kb fragment isolated by agarose gel purification. T16-4.5 was cleaved with Acc I and Hpa I and the 0.65 kb band was also isolated by agarose gel purification. The two agarose gel purified restriction fragments and the dephosphorylated pKK-CFTR1 were ligated to produce pKK-CFTR2. Alternatively, pKK-CFTR2 could have been constructed using corresponding restriction fragments from the partial CFTR cDNA clone C1-1/5. pKK-CFTR2 contains the uninterrupted CFTR protein coding sequence and conferred slow growth upon *E. coli* host cells in which it was inserted, whereas pKK-CFTR1 did not. The origin of replication of pKK-CFTR2 is derived from pMB1 and confers a plasmid copy number of 15-20 copies per host cell.

2145641

Example 2 - Improving Host Cell Viability

An additional enhancement of host cell viability was accomplished by a further reduction in the copy number of CFTR cDNA per host cell. This was achieved by transferring the CFTR cDNA into the plasmid vector, pSC-3Z. pSC-3Z was constructed using the pSC101 replication origin of the low copy number plasmid pLG338 (Stoker *et al.*, Gene 18, 335 (1982)) and the ampicillin resistance gene and polylinker of pGEM-3Z (available from Promega). pLG338 was cleaved with Sph I and Pvu II and the 2.8 kb fragment containing the replication origin isolated by agarose gel purification. pGEM-3Z was cleaved with Alw NI, the resultant restriction fragment ends treated with T4 DNA polymerase and deoxynucleotide triphosphates, cleaved with Sph I and the 1.9 kb band containing the ampicillin resistance gene and the polylinker was isolated by agarose gel purification. The pLG338 and pGEM-3Z fragments were ligated together to produce the low copy number cloning vector pSC-3Z. pSC-3Z and other plasmids containing pSC101 origins of replication are maintained at approximately five copies per cell (Sambrook *et al. supra*).

With additional reference to Figure 4, pKK-CFTR2 was cleaved with Eco RV, Pst I and Sal I and then passed over a Sephacryl S400 spun column (available from Pharmacia) according to the manufacturer's procedure in order to remove the Sal I to Eco RV restriction fragment which was retained within the column. pSC-3Z was digested with Sma I and Pst I and also passed over a Sephacryl S400 spun column to remove the small Sma I/Pst I restriction fragment which was retained within the column. The column eluted fractions from the pKK-CFTR2 digest and the pSC-3Z digest were mixed and ligated to produce pSC-CFTR2. A map of this plasmid is presented in Figure 5. Host cells containing CFTR cDNAs at this and similar gene dosages grow well and have stably maintained the recombinant plasmid with the full length CFTR coding sequence. In addition, this plasmid contains a bacteriophage T7 RNA polymerase promoter adjacent to the CFTR coding sequence and is therefore convenient for *in vitro* transcription/translation of the CFTR protein. The nucleotide sequence of CFTR coding region from pSC-CFTR2 plasmid is presented in Sequence Listing 1 as SEQ ID NO:1. Significantly, this sequence differs from the previously published (Riordan, J.R. *et al.* (1989) *Science* 245:1066-1073) CFTR sequence at position 1990, where there is C in place of the reported A. See Gregory, R.J. *et al.* (1990) *Nature* 347:382-386. *E. coli* host cells containing pSC-CFTR2, internally identified with the number pSC-CFTR2/AG1, have been deposited at the American Type Culture Collection and given the accession number: ATCC 68244.

Example 3 - Alternate Method for Improving Host Cell Viability

A second method for enhancing host cell viability comprises disruption of the CFTR protein coding sequence. For this purpose, a synthetic intron was designed for insertion between nucleotides 1716 and 1717 of the CFTR cDNA. This intron is especially

advantageous because of its easily manageable size. Furthermore, it is designed to be efficiently spliced from CFTR primary RNA transcripts when expressed in eukaryotic cells. Four synthetic oligonucleotides were synthesized (1195RG, 1196RG, 1197RG and 1198RG) collectively extending from the Sph I cleavage site at position 1700 to the Hinc II cleavage site at position 1785 and including the additional 83 nucleotides between 1716 and 1717 (see Figure 6). These oligonucleotides were phosphorylated with T4 polynucleotide kinase as described by Sambrook et al., mixed together, heated to 95°C for 5 minutes in the same buffer used during phosphorylation, and allowed to cool to room temperature over several hours to allow annealing of the single stranded oligonucleotides. To insert the synthetic intron into the CFTR coding sequence and with reference to Figures 7A and 7B, a subclone of plasmid T11 was made by cleaving the Sal I site in the polylinker, repairing the recessed ends of the cleaved DNA with deoxynucleotide triphosphates and the large fragment of DNA Polymerase I and religating the DNA. This plasmid was then digested with Eco RV and Nru I and religated. The resulting plasmid T16-Δ5' extended from the Nru I site at position 490 of the CFTR cDNA to the 3' end of clone T16 and contained single sites for Sph I and Hinc II at positions corresponding to nucleotides 1700 and 1785 of the CFTR cDNA. T16-Δ5' plasmid was cleaved with Sph I and Hinc II and the large fragment was isolated by agarose gel purification. The annealed synthetic oligonucleotides were ligated into this vector fragment to generate T16-intron.

T16-intron was then digested with Eco RI and Sma I and the large fragment was isolated by agarose gel purification. T16-4.5 was digested with Eco RI and Sca I and the 790 bp fragment was also isolated by agarose gel purification. The purified T16-intron and T16-4.5 fragments were ligated to produce T16-intron-2. T16-intron-2 contains CFTR cDNA sequences extending from the Nru I site at position 490 to the Sca I site at position 2818, and includes the unique Hpa I site at position 2463 which is not present in T16-1 or T16-intron-1.

T16-intron-2 was then cleaved with Xba I and Hpa I and the 1800 bp fragment was isolated by agarose gel purification. pKK-CFTR1 was digested with Xba I and Hpa I and the large fragment was also isolated by agarose gel purification and ligated with the fragment derived from T16-intron-2 to yield pKK-CFTR3, shown in Figure 8. The CFTR cDNA within pKK-CFTR3 is identical to that within pSC-CFTR2 and pKK-CFTR2 except for the insertion of the 83 bp intron between nucleotides 1716 and 1717. The insertion of this intron resulted in improved growth characteristics for cells harboring pKK-CFTR3 relative to cells containing the unmodified CFTR cDNA in pKK-CFTR2.

Example 4 - In vitro Transcription/Translation

In addition to sequence analysis, the integrity of the CFTR cDNA open reading frame was verified by *in vitro* transcription/translation. This method also provided the initial CFTR protein for identification purposes. 5 micrograms of pSC-CFTR2 plasmid DNA were linearized with Sal I and used to direct the synthesis of CFTR RNA transcripts with T7 RNA

polymerase as described by the supplier (Stratagene). This transcript was extracted with phenol and chloroform and precipitated with ethanol. The transcript was resuspended in 25 microliters of water and varying amounts were added to a reticulocyte lysate *in vitro* translation system (Promega). The reactions were performed as described by the supplier in the presence of canine pancreatic microsomal membranes (Promega), using ³⁵S-methionine to label newly synthesized proteins. *In vitro* translation products were analysed by discontinuous polyacrylamide gel electrophoresis in the presence of 0.1% SDS with 8% separating gels (Laemmli, U.K. (1970) *Nature* 227:680-685). Before electrophoresis, the *in vitro* translation reactions were denatured with 3% SDS, 8 M urea and 5% 2-mercaptoethanol in 0.65 M Tris-HCl, pH 6.8. Following electrophoresis, the gels were fixed in methanol:acetic acid:water (30:10:60), rinsed with water and impregnated with 1 M sodium salicylate. ³⁵S labelled proteins were detected by fluorography. A band of approximately 180 kD was detected, consistent with translation of the full length CFTR insert.

15

Example 5 - Elimination of Cryptic Regulatory Signals

Analysis of the DNA sequence of the CFTR has revealed the presence of a potential *E. coli* RNA polymerase promoter between nucleotides 748 and 778 which conforms well to the derived consensus sequence for *E. coli* promoters (Reznikoff and McClure, Maximizing Gene Expression, 1, Butterworth Publishers, Stoneham, MA). If this sequence functions as a promoter functions in *E. coli*, it could direct synthesis of potentially toxic partial CFTR polypeptides. Thus, an additional advantageous procedure for maintaining plasmids containing CFTR cDNAs in *E. coli* would be to alter the sequence of this potential promoter such that it will not function in *E. coli*. This may be accomplished without altering the amino acid sequence encoded by the CFTR cDNA. Specifically, plasmids containing complete or partial CFTR cDNA's would be altered by site-directed mutagenesis using synthetic oligonucleotides (Zoller and Smith, (1983) *Methods Enzymol.* 100:468). More specifically, altering the nucleotide sequence at position 908 from a T to C and at position 774 from an A to a G effectively eliminates the activity of this promoter sequence without altering the amino acid coding potential of the CFTR open reading frame. Other potential regulatory signals within the CFTR cDNA for transcription and translation could also be advantageously altered and/or deleted by the same method.

Further analysis has identified a sequence extending from nucleotide 908 to 936 which functions efficiently as a transcriptional promoter element in *E. coli* (Gregory, R.J. et al. (1990) *Nature* 347:382-386). Mutation at position 936 is capable of inactivating this promoter and allowing the CFTR cDNA to be stably maintained as a plasmid in *E. coli* (Cheng, S.H. et al. (1990) *Cell* 63:827-834). Specifically position 936 has been altered from a C to a T residue without the amino acid sequence encoded by the cDNA being altered. Other mutations within this regulatory element described in Gregory, R.J. et al. (1990)

Nature 347:382-386 could also be used to inactivate the transcriptional promoter activity. Specifically, the sequence from 908 to 913 (TTGTGA) and from 931 to 936 (GAAAAT) could be altered by site directed mutagenesis without altering the amino acid sequence encoded by the cDNA.

5

Example 6 - Cloning of CFTR in Alternate Host Systems

Although the CFTR cDNA displays apparent toxicity in *E. coli* cells, other types of host cells may not be affected in this way. Alternative host systems in which the entire CFTR cDNA protein encoding region may be maintained and/or expressed include other
10 bacterial species and yeast. It is not possible *a priori* to predict which cells might be resistant and which might not. Screening a number of different host/vector combinations is necessary to find a suitable host tolerant of expression of the full length protein or potentially toxic fragments thereof.

15

Example 7 - Generation of Adenovirus Vector Encoding CFTR (Ad2/CFTR)

1. DNA preparation - Construction of the recombinant Ad2/CFTR-1 virus (the sequence of which is shown in Table II and as SEQ ID NO:3) was accomplished as follows: The CFTR cDNA was excised from the plasmid pCMV-CFTR-936C using restriction enzymes
20 SpeI and EclI361. pCMV-CFTR-936C consists of a minimal CFTR cDNA encompassing nucleotides 123-4622 of the published CFTR sequence cloned into the multiple cloning site of pRC/CMV (Invitrogen Corp.) using synthetic linkers. The CFTR cDNA within this plasmid has been completely sequenced. The SpeI/EclI361 restriction fragment contains 47 bp of 5' sequence derived from synthetic linkers and the multiple cloning site of the vector.

25

The CFTR cDNA (the sequence of which is shown as SEQ ID NO:1 and the amino acid sequence encoded by the CFTR cDNA is shown as SEQ ID NO:2) was inserted between the NheI and SnaBI restriction sites of the adenovirus gene transfer vector pBR-Ad2-7. pBR-Ad2-7 is a pBR322 based plasmid containing an approximately 7 kb insert derived from the 5' 10680 bp of Ad2 inserted between the Clal and BamHI sites of pBR322. From this Ad2
30 fragment, the sequences corresponding to Ad2 nucleotides 546-3497 were deleted and replaced with a 12 bp multiple cloning site containing an NheI site, an MluI site, and a SnaBI site. The construct also contains the 5' inverted terminal repeat and viral packaging signals, the Ela enhancer and promoter, the Elb 3' intron and the 3' untranslated region and polyadenylation sites. The resulting plasmid was called pBR-Ad2-7/CFTR. Its use to
35 assemble virus is described below.

2. Virus Preparation from DNA - To generate the recombinant Ad2/CFTR-1 adenovirus, the vector pBR-Ad2-7/CFTR was cleaved with BstBI at the site corresponding to the unique BstBI site at 10670 in Ad2. The cleaved plasmid DNA was ligated to BstBI restricted Ad2

2145641

- 27 -

DNA. Following ligation, the reaction was used to transfect 293 cells by the calcium phosphate procedure. Approximately 7-8 days following transfection, a single plaque appeared and was used to reinfect a dish of 293 cells. Following development of cytopathic effect (CPE), the medium was removed and saved. Total DNA was prepared from the infected cells and analyzed by restriction analysis with multiple enzymes to verify the integrity of the construct. Viral supernatant was then used to infect 293 cells and upon development of CPE, expression of CFTR was assayed by the protein kinase A (PKA) immunoprecipitation assay (Gregory, R.J. et al. (1990) *Nature* 347:382). Following these verification procedures, the virus was further purified by two rounds of plaque purification.

Plaque purified virus was grown into a small seed stock by inoculation at low multiplicities of infection onto 293 cells grown in monolayers in 925 medium supplemented with 10% bovine calf serum. Material at this stage was designated a Research Viral Seed Stock (RVSS) and was used in all preliminary experiments.

3. Virus Host Cell - Ad2/CFTR-1 is propagated in human 293 cells (ATCC CRL 1573). These cells are a human embryonal kidney cell line which were immortalized with sheared fragments of human Ad5 DNA. The 293 cell line expresses adenovirus early region 1 gene products and in consequence, will support the growth of E1 deficient adenoviruses. By analogy with retroviruses, 293 cells could be considered a packaging cell line, but they differ from usual retrovirus lines in that they do not provide missing viral structural proteins, rather, they provide only some missing viral early functions.

Production lots of virus are propagated in 293 cells derived from the Working Cell Bank (WCB). The WCB is in turn derived from the Master Cell Bank (MCB) which was grown up from a fresh vial of cells obtained from ATCC. Because 293 cells are of human origin, they are being tested extensively for the presence of biological agents. The MCB and WCB are being characterized for identity and the absence of adventitious agents by Microbiological Associates, Rockville, MD.

4. Growth of Production Lots of Virus

Production lots of Ad2/CFTR-1 are produced by inoculation of approximately $5-10 \times 10^7$ pfu of MVSS onto approximately $1-2 \times 10^7$ Wcb 293 cells grown in a T175 flask containing 25 mls of 925 medium. Inoculation is achieved by direct addition of the virus (approximately 2-5 mls) to each flask. Batches of 50-60 flasks constitute a lot.

Following 40-48 hours incubation at 37°C, the cells are shaken loose from the flask and transferred with medium to a 250 ml centrifuge bottle and spun at 1000 xg. The cell pellet is resuspended in 4 ml phosphate buffered saline containing 0.1 g/l CaCl_2 and 0.1g/l MgCl_2 and the cells subjected to cycles of freeze-thaw to release virus. Cellular debris is removed by centrifugation at 1000 xg for 15 min. The supernatant from this centrifugation is layered on top of the CsCl step gradient: 2 ml 1.4g/ml CsCl and 3 ml 1.25g/ml CsCl in 10

mM Tris, 1 mM EDTA (TE) and spun for 1 hour at 35,000 rpm in a Beckman SW41 rotor. Virus is then removed from the interface between the two CsCl layers, mixed with 1.35 g/ml CsCl in TE and then subjected to a 2.5 hour equilibrium centrifugation at 75,000 rpm in a TLN-100 rotor. Virus is removed by puncturing the side of the tube with a hypodermic
5 needle and gently removing the banded virus. To reduce the CsCl concentration, the sample is dialyzed against 2 changes of 2 liters of phosphate buffered saline with 10% sucrose.

Following this procedure, dialyzed virus is stable at 4°C for several weeks or can be stored for longer periods at -80°C. Aliquots of material for human use will be tested and while awaiting the results of these tests, the remainder will be stored frozen. The tests to be
10 performed are described below:

5. Structure and Purity of Virus

SDS polyacrylamide gel electrophoresis of purified virions reveals a number of polypeptides, many of which have been characterized. When preparations of virus were
15 subjected to one or two additional rounds of CsCl centrifugation, the protein profile obtained was indistinguishable. This indicates that additional equilibrium centrifugation does not purify the virus further, and may suggest that even the less intense bands detected in the virus preparations represent minor virion components rather than contaminating proteins. The identity of the protein bands is presently being established by N-terminal sequence analysis.

6. Contaminating Materials - The material to be administered to patients will be 2×10^6 pfu, 2×10^7 pfu and 5×10^7 pfu of purified Ad2/CFTR-1. Assuming a minimum particle to pfu ratio of 500, this corresponds to 1×10^9 , 1×10^{10} and 2.5×10^{10} viral particles, these
25 correspond to a dose by mass of 0.25 µg, 2.5 µg and 6.25 µg assuming a molecular mass for adenovirus of 150×10^6 .

The origin of the materials from which a production lot of the purified Ad2/CFTR-1 is derived was described in detail above and is illustrated as a flow diagram in Figure 6. All the starting materials from which the purified virus is made (i.e., MCB, and WCB, and the MVSS) will be extensively tested. Further, the growth medium used will be tested and the
30 serum will be from only approved suppliers who will provide test certificates. In this way, all the components used to generate a production lot will have been characterized. Following growth, the production lot virus will be purified by two rounds of CsCl centrifugation, dialyzed, and tested. A production lot should constitute $1-5 \times 10^{10}$ pfu Ad2/CFTR-1.

As described above, to detect any contaminating material aliquots of the production
35 lot will be analyzed by SDS gel electrophoresis and restriction enzyme mapping. However, these tests have limited sensitivity. Indeed, unlike the situation for purified single chain recombinant proteins, it is very difficult to quantitate the purity of the AD2/CFTR-1 using SDS polyacrylamide gel electrophoresis (or similar methods). An alternative is the immunological detection of contaminating proteins (IDCP). Such an assay utilizes antibodies

2145641

- 29 -

raised against the proteins purified in a mock purification run. Development of such an assay has not yet been attempted for the CsCl purification scheme for Ad2/CFTR-1. However, initially an IDCP assay developed for the detection of contaminants in recombinant proteins produced in Chinese hamster ovary (CHO) cells will be used. In addition, to hamster
5 proteins, these assays detect bovine serum albumin (BSA), transferrin and IgG heavy and light chain derived from the serum added to the growth medium. Tests using such reagents to examine research batches of Ad2/CFTR-1 by both ELISA and Western blots are in progress.

Other proteins contaminating the virus preparation are likely to be from the 293 cells - that is, of human origin. Human proteins contaminating therapeutic agents derived from
10 human sources are usually not problematic. In this case, however, we plan to test the production lot for transforming factors. Such factors could be activities of contaminating human proteins or of the Ad2/CFTR-1 vector or other contaminating agents. For the test, it is proposed that 10 dishes of Rat 1 cells containing 2×10^6 cells (the number of target cells in the patient) with 4 times the highest human dose of Ad2/CFTR-1 (2×10^8 pfu) will be
15 infected. Following infection, the cells will be plated out in agar and examined for the appearance of transformed foci for 2 weeks. Wild type adenovirus will be used as a control.

Nucleic acids and proteins would be expected to be separated from purified virus preparations upon equilibrium density centrifugation. Furthermore, the 293 cells are not expected to contain VL30 sequences. Biologically active nucleic cells should be detected.
20

Example 8 - Preliminary Experiments Testing the Ability of Ad2/ β Gal or Ad2/CFTR Virus to Enter Airway Epithelial Cells

a. Hamster Studies

25 Initial studies involving the intratracheal instillation of the Ad- β Gal viral vector into Syrian hamsters, which are reported to be permissive for human adenovirus are being performed. The first study, a time course assessment of the pulmonary and systemic acute inflammatory response to a single intratracheal administration of Ad- β Gal viral vector, has been completed. In this study, a total of 24 animals distributed among three treatment
30 groups, specifically, 8 vehicle control, 8 low dose virus (1×10^{11} particles; 3×10^8 pfu), and 8 high dose virus (1.7×10^{12} particles; 5×10^9 pfu), were used. Within each treatment group, 2 animals were analyzed at each of four time points after viral vector instillation: 6 hrs, 24 hrs, 48 hrs, and 7 days. At the time of sacrifice of each animal, lung lavage and blood samples were taken for analysis. The lungs were fixed and processed for normal light-level
35 histology. Blood and lavage fluid were evaluated for total leukocyte count and leukocyte differential. As an additional measure of the inflammatory process, lavage fluid was also evaluated for total protein. Following embeddings, sectioning and hematoxylin/eosin staining, lung sections were evaluated for signs of inflammation and airway epithelial damage.

With the small sample size, the data from this preliminary study were not amenable to statistical analyses, however, some general trends could be ascertained. In the peripheral blood samples, total leukocyte counts showed no apparent dose- or time- dependent changes. In the blood leukocyte differential counts, there may have been a minor dose-related
5 elevation in percent neutrophil at 6 hours; however, data from all other time points showed no elevation in neutrophil percentages. Taken together, these data suggest little or nor systemic inflammatory response to the viral administration.

From the lung lavage, some elevation in total neutrophil counts were observed at the first three time points (6 hr, 24 hr, 48 hr). By seven days, both total and percent neutrophil
10 values had returned to normal range. The trends in lung lavage protein levels were more difficult to assess due to inter-animal variability; however, no obvious dose- or time- dependent effects were apparent. First, no damage to airway epithelium was observed at any time point or virus dose level. Second, a time- and dose- dependent mild inflammatory response was observed, being maximal at 48 hr in the high virus dose animals. By seven
15 days, the inflammatory response had completely resolved, such that the lungs from animals in all treatment groups were indistinguishable.

In summary, a mild, transient, pulmonary inflammatory response appears to be associated with the intratracheal administration of the described doses of adenoviral vector in the Syrian Hamster.

A second, single intratracheal dose, hamster study has been initiated. This study is designed to assess the possibility of the spread of ineffective viral vectors to organs outside of the lung and the antibody response of the animals to the adenoviral vector. In this study, the three treatment groups (vehicle control, low dose virus, high dose virus) each contained 12 animals. Animals will be evaluated at three time points: 1 day, 7 days, and 1 month. In this
20 study, viral vector persistence and possible spread will be evaluated by the assessment of the presence of infective virions in numerous organs including lung, gut, heart, liver, spleen, kidney, brain and gonads. Changes in adenoviral antibody titer will be measured in peripheral blood and lung lavage. Additionally, lung lavage, peripheral blood and lung histology will be evaluated as in the previous study.
25

30
b. Primate studies.

Studies of recombinant adenovirus are also underway in primates. The goal of these studies is to assess the ability of recombinant adenoviral vectors to deliver genes to the respiratory epithelium *in vivo* and to assess the safety of the construct in primates. Initial
35 studies in primates targeted nasal epithelia as the site of infection because of its similarity to lower airway epithelia, because of its accessibility, and because nasal epithelia was used for the first human studies. The Rhesus monkey (*Macaca mulatta*) has been chosen for studies, because it has a nasal epithelium similar to that of humans.

2145641

- 31 -

How expression of CFTR affects the electrolyte transport properties of the nasal epithelium can be studied in patients with cystic fibrosis. But because the primates have normal CFTR function, instead the ability to transfer a reporter gene was assessed. Therefore the Ad- β Gal virus was used. The epithelial cell density in the nasal cavity of the Rhesus monkey is estimated to be 2×10^6 cells/cm (based on an average nasal epithelial cell diameter of 7 μ m) and the surface near 25-50 cm². Thus, there are about 5×10^7 cells in the nasal epithelium of Rhesus monkey. To focus especially on safety, the higher viral doses (20-200 MOI) were used *in vivo*. Thus doses in the range of 10^9 - 10^{10} pfu were used.

In the first pilot study the right nostril of Monkey A was infected with Ad- β -Gal (~1 ml). This viral preparation was purified by CsCl gradient centrifugation and then by gel filtration chromatography one week later. Adenoviruses are typically stable in CsCl at 4°C for one to two weeks. However, this viral preparation was found to be defective (i.e., it did not produce detectable β -galactosidase activity in the permissive 293 cells). Thus, it was concluded that there was no live viral activity in the material. β -galactosidase activity in nasal epithelial cells from Monkey A was also not detected. Therefore, in the next study, two different preparations of Ad- β -Gal virus: one that was purified on a CsCl gradient and then dialyzed against Tris-buffered saline to remove the CsCl, and a crude unpurified one was used. Titers of Ad- β -Gal viruses were $\sim 2 \times 10^{10}$ pfu/ml and $> 1 \times 10^{13}$ pfu/ml, respectively, and both preparations produced detectable β -galactosidase activity in 293 cells.

Monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg). One week before administration of virus, the nasal mucosa of each monkey was brushed to establish baseline cell differentials and levels of β -galactosidase. Blood was drawn for baseline determination of cell differentials, blood chemistries, adenovirus antibody titers, and viral cultures. Each monkey was also examined for weight, temperature, appetite, and general health prior to infection.

The entire epithelium of one nasal cavity was used in each monkey. A foley catheter (size 10) was inserted through each nasal cavity into the pharynx, inflated with 2-3 ml of air, and then pulled anteriorly to obtain tight posterior occlusion at the posterior choana. Both nasal cavities were then irrigated with a solution (~5 ml) of 5 mM dithiothreitol plus 0.2 U/ml neuraminidase in phosphate-buffered saline (PBS) for five minutes. This solution was used to dissolve any residual mucus overlaying the epithelia. (It was subsequently found that such treatment is not required.) The washing procedure also allowed the determination of whether the balloons were effectively isolating the nasal cavity. The virus (Ad- β -Gal) was then slowly instilled into the right nostril with the posterior balloon inflated. The viral solution remained in contact with the nasal mucosa for 30 minutes. At the end of 30 minutes, the remaining viral solution was removed by suction. The balloons were deflated, the catheters removed, and the monkey allowed to recover from anesthesia. Monkey A received the CsCl-purified virus (~1.5 ml) and Monkey B received the crude virus (~6 ml). (note that this was the second exposure of Monkey A to the recombinant adenovirus).

Both monkeys were followed daily for appearance of the nasal mucosa, conjunctivitis, appetite, activity, and stool consistency. Each monkey was subsequently anesthetized on days 1, 4, 7, 14, and 21 to obtain nasal, pharyngeal, and tracheal cell samples (either by swabs or brushes) as described below. Phlebotomy was performed over the same time course for hematology, ESR, general screen, antibody serology and viral cultures. Stools were collected every week to assess viral cultures.

To obtain nasal epithelial cells from an anesthetized monkey, the nasal mucosa was first impregnated with 5 drops of Afrin (0.05% oxymetazoline hydrochloride, Schering-Plough) and 1 ml of 2% Lidocaine for 5 min. A cytobrush (the kind typically used for Pap smears) was then used to gently rub the mucosa for about 10 seconds. For tracheal brushings, a flexible fiberoptic bronchoscope; a 3 mm cytology brush (Bard) was advanced through the bronchoscope into the trachea, and a small area was brushed for about 10 seconds. This procedure was repeated twice to obtain a total of $\sim 10^6$ cells/ml. Cells were then collected on slides (approximately 2×10^4 cells/slide using a Cytospin 3 (Shandon, PA)) for subsequent staining (see below).

To determine viral efficacy, nasal, pharyngeal, and tracheal cells were stained for β -galactosidase using X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside). Cleavage of X-gal by β -galactosidase produces a blue color that can be seen with light microscopy. The Ad- β -gal vector included a nuclear-localization signal (NLS) (from SV40 large T-antigen) at the amino-terminus of the β -galactosidase sequence to direct expression of this protein to the nucleus. Thus, the number of blue nuclei after staining was determined.

RT-PCR (reverse transcriptase-polymerase chain reaction) was also used to determine viral efficacy. This assay indicates the presence of β -galactosidase mRNA in cells obtained by brushings or swabs. PCR primers were used in both the adenovirus sequence and the LacZ sequence to distinguish virally-produced mRNA from endogenous mRNA. PCR was also used to detect the presence of the recombinant adenovirus DNA. Cytospin preparations were used to assess for the presence of virally produced β -galactosidase mRNA in the respiratory epithelial cells using *in-situ* hybridization. This technique has the advantage of being highly specific and will allow assessment which cells are producing the mRNA.

Whether there was any inflammatory response was assessed by visual inspection of the nasal epithelium and by cytological examination of Wright-stained cells (cytospin). The percentage of neutrophils and lymphocytes were compared to that of the control nostril and to the normal values from four control monkeys. Systemic responses by white blood cell counts, sedimentation rate, and fever were also assessed.

Viral replication at each of the time points was assessed by testing for the presence of live virus in the supernatant of the cell suspension from swabs or brushes. Each supernatant was used to infect (at several dilutions) the virus-sensitive 293 cell line. Cytopathic changes in the 293 cells were monitored for 1 week and then the cells were fixed and stained for β -galactosidase. Cytopathic effects and blue-stained cells indicated the presence of live virus.

2145641

Positive supernatants will also be subjected to analysis of nonintegrating DNA to identify (confirm) the contributing virus(es).

Antibody titers to type 2 adenovirus and to the recombinant adenovirus were determined by ELISA. Blood/serum analysis was performed using an automated chemistry analyzer Hitachi 737 and an automated hematology analyzer Technicom H6. The blood buffy coat was cultured in A549 cells for wild type adenovirus and was cultured in the permissive 293 cells.

Results: Both monkeys tolerated the procedure well. Daily examination revealed no evidence of coryza, conjunctivitis or diarrhea. For both monkeys, the nasal mucosa was mildly erythematous in both the infection side and the control side; this was interpreted as being due to the instrumentation. Appetites and weights were not affected by virus administered in either monkey. Physical examination on days 1, 4, 7, 14 and 21 revealed no evidence of lymphadenopathy, tachypnea, or tachycardia. On day 21, monkey B had a temperature 39.1°C (normal for Rhesus monkey 38.8°C) but had no other abnormalities on physical exam or in laboratory data. Monkey A had a slight leukocytosis on day 1 post infection which returned to normal by day 4; the WBC was 4,920 on the day of infection, 8,070 on day 1, and 5,200 on day 4. The ESR did not change after the infection. Electrolytes and transaminases were normal throughout.

Wright stains of cells from nasal brushing were performed on days 4, 7, 14, and 21. They revealed less than 5% neutrophils and lymphocytes. There was no difference between the infected and the control side.

X-Gal stains of the pharyngeal swabs revealed blue-stained cells in both monkeys on days 4, 7, and 14; only a few of the cells had clear nuclear localization of the pigment and some pigment was seen in extracellular debris. On day 7 post infection, X-Gal stains from the right nostril of monkey A, revealed a total of 135 ciliated cells with nuclear-localized blue stain. The control side had only 4 blue cells. Monkey B had 2 blue cells from the infected nostril and none from the control side. Blue cells were not seen on day 7, 14, or 21.

RT-PCR on day 3 post infection revealed a band of the correct size that hybridized with a β -Gal probe, consistent with β -Gal mRNA in the samples from Monkey A control nostril and Monkey B infected nostril. On day 7 there was a positive band in the sample from the infected nostril of Monkey A, the same specimen that revealed blue cells.

Fluid from each nostril, the pharynx, and trachea of both monkeys was placed on 293 cells to check for the presence of live virus by cytopathic effect and X-Gal stain. In Monkey A, live virus was detected in both nostrils on day 3 after infection; no live virus was detected at either one or two weeks post-infection. In Monkey B, live virus was detected in both nostrils, pharynx, and trachea on day 3, and only in the infected nostril on day 7 after infection. No live virus was detected 2 weeks after the infection.

c. Human Explant Studies

In a second type of experiment, epithelial cells from a nasal polyp of a CF patient were cultured on permeable filter supports. These cells form an electrically tight epithelial monolayer after several days in culture. Eight days after seeding, the cells were exposed to the Ad2/CFTR virus for 6 hours. Three days later, the short-circuit current (I_{sc}) across the monolayer was measured. cAMP agonists did not increase the I_{sc}, indicating that there was no change in chloride secretion. However, this defect was corrected after infection with recombinant Ad2/CFTR. Cells infected with Ad2/CFTR (MOI=5; MOI refers to multiplicity of infection; 1 MOI indicates one pfu/cell) express functional CFTR; cAMP agonists stimulated I_{sc}, indicating stimulation of Cl⁻ secretion. Ad2/CFTR also corrected the CF chloride channel defect in CF tracheal epithelial cells. Additional studies indicated that Ad2/CFTR was able to correct the chloride secretory defect without altering the transepithelial electrical resistance; this result indicates that the integrity of the epithelial cells and the tight junctions was not disrupted by infection with Ad2/CFTR. Application of 1 MOI of Ad2/CFTR was also found to be sufficient to correct the CF chloride secretory defect.

The experiments using primary cultures of human airway epithelial cells indicate that the Ad2/CFTR virus is able to enter CF airway epithelial cells and express sufficient CFTR to correct the defect in chloride transport.

20 Example 9 -In Vivo Delivery to and Expression of CFTR in Cotton Rat and Rhesus Monkey Epithelium

MATERIALS AND METHODS

Adenovirus vector

25 Ad2/CFTR-1 was prepared as described in Example 7. The DNA construct comprises a full length copy of the Ad2 genome of approximately 37.5 kb from which the early region 1 genes (nucleotides 546 to 3497) have been replaced by cDNA for CFTR (nucleotides 123 to 4622 of the published CFTR sequence with 53 additional linker nucleotides). The viral Ela promoter was used for CFTR cDNA. Termination/polyadenylation occurs at the site normally used by the Elb and protein IX transcripts. The recombinant virus E3 region was conserved. The size of the Ad2-CFTR-1 vector is approximately 104.5% that of wild-type adenovirus. The recombinant virus was grown in 293 cells that complement the E1 early viral promoters. The cells were frozen and thawed three times to release the virus and the preparation was purified on a CsCl gradient, then dialyzed against Tris-buffered saline (TBS) to remove the CsCl, as described.

2145641

- 35 -

Animals

Rats. Twenty two cotton rats (6-8 weeks old, weighing between 80-100 g) were used for this study. Rats were anesthetized by inhaled methoxyflurane (Pitman Moore, Inc., Mundelen, Ill). Virus was applied to the lungs by nasal instillation during inspiration.

5 Two cotton rat studies were performed. In the first study, seven rats were assigned to a one time pulmonary infection with 100 μ l solution containing 4.1×10^9 plaque forming units (pfu) of the Ad2/CFTR-1 virus and 3 rats served as controls. One control rat and either two or three experimental rats were sacrificed with methoxyflurane and studies at each of three time points: 4, 11, or 15 days after infection.

10 The second group of rats was used to test the effect of repeat administration of the recombinant virus. All 12 rats received 2.1×10^8 pfu of the Ad2/CFTR-1 virus on day 0 and 9 of the rats received a second dose of 3.2×10^8 pfu of Ad2/CFTR-1 14 days later. Groups of one control rat and three experimental rats were sacrificed at 3, 7, or 14 days after the second administration of virus. Before necropsy, the trachea was cannulated and
15 bronchoalveolar lavage (BAL) was performed with 3 ml aliquots of phosphate-buffered saline. A median sternotomy was performed and the right ventricle cannulated for blood collection. The right lung and trachea were fixed in 4% formaldehyde and the left lung was frozen in liquid nitrogen and kept at -70°C for evaluation by immunochemistry, reverse transcriptase polymerase chain reaction (RT-PCR), and viral culture. Other organs were removed and
20 quickly frozen in liquid nitrogen for evaluation by polymerase chain reaction (PCR).

Monkeys. Three female Rhesus monkeys were used for this study; a fourth female monkey was kept in the same room, and was used as control. For application of the virus, the monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg). The entire epithelium of one nasal cavity in each monkey was used for virus application. A foley
25 catheter (size 10) was inserted through each nasal cavity into the pharynx, the balloon was inflated with 2-3 ml of air, and then pulled anteriorly to obtain a tight occlusion at the posterior choana. The Ad2/CFTR-1 virus was then instilled slowly in the right nostril with the posterior balloon inflated. The viral solution remained in contact with the nasal mucosa for 30 min. The balloons were deflated, the catheters were removed, and the monkeys were
30 allowed to recover from anesthesia. A similar procedure was performed on the left nostril, except that TBS solution was instilled as a control. The monkeys received a total of three doses of the virus over a period of 5 months. The total dose given was 2.5×10^9 pfu the first time, 2.3×10^9 pfu the second time, and 2.8×10^9 pfu the third time. It was estimated that the cell density of the nasal epithelia to be 2×10^6 cells/cm² and a surface area of 25 to 50
35 cm². This corresponds to a multiplicity of infection (MOI) of approximately 25.

The animals were evaluated 1 week before the first administration of virus, on the day of administration, and on days 1, 3, 6, 13, 21, 27, and 42 days after infection. The second administration of virus occurred on day 55. The monkeys were evaluated on day 55 and then on days 56, 59, 62, 69, 76, 83, 89, 96, 103, and 111. For the third administration, on day 134.

only the left nostril was cannulated and exposed to the virus. The control monkey received instillations of PBS instead of virus. Biopsies of the left medial turbinate were carried out on day 135 in one of the infected monkeys, on day 138 on the second infected monkey, and on day 142 on the third infected monkey and on the control monkey.

- 5 For evaluations, monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg). To obtain nasal epithelial cells, the nasal mucosa was first impregnated with 5 drops of Afrin (0.05% oxymetazoline hydrochloride, Schering-Plough) and 1 ml of 2% Lidocaine for 5 minutes. A cytobrush was then used to gently rub the mucosa for about 3 sec. To obtain pharyngeal epithelial swabs, a cotton-tipped applicator was rubbed over the back of the pharynx 2-3 times. The resulting cells were dislodged from brushes or
10 applicators into 2 ml of sterile PBS. Biopsies of the medial turbinate were performed using cupped forceps under direct endoscopic control.

- Animals were evaluated daily for evidence of abnormal behavior of physical signs. A record of food and fluid intake was used to assess appetite and general health. Stool
15 consistency was also recorded to check for the possibility of diarrhea. At each of the evaluation time points, rectal temperature, respiratory rate, and heart rate were measured. The nasal mucosa, conjunctivas, and pharynx were visually inspected. The monkeys were also examined for lymphadenopathy.

- Venous blood from the monkeys was collected by standard venipuncture technique.
20 Blood/serum analysis was performed in the clinical laboratory of the University of Iowa Hospitals and Clinics using a Hitachi 737 automated chemistry analyzer and a Technicom H6 automated hematology analyzer.

Serology

- 25 Sera were obtained and anti-adenoviral antibody titers were measured by an enzyme-linked immunoabsorbant assay (ELISA). For the ELISA, 50 ng/well of filled adenovirus (Lee Biomolecular Research Laboratories, San Diego, Ca) in 0.1M NaHCO₃ were coated on 96 well plates at 4°C overnight. The test samples at appropriate dilutions were added, starting at a dilution of 1/50. The samples were incubated for 1 hour, the plates washed, and
30 a goat anti-human IgG HRP conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 1 hour. The plates were washed and O-Phenylenediamine (Sigma Chemical Co., St. Louis, MO) was added for 30 min. at room temperature. The assay was stopped with 4.5 M H₂SO₄ and read at 490 nm on a Molecular Devices microplate reader. The titer was calculated as the product of the reciprocal of the initial dilution and the
35 reciprocal of the dilution in the last well with an OD>0.100.

Neutralizing antibodies measure the ability of the monkey serum to prevent infection of 293 cells by adenovirus. Monkey serum (1:25 dilution) [or nasal washings (1:2 dilutions)] was added in two-fold serial dilutions to a 96 well plate. Adenovirus (2.5 x 10⁵ pfu) was added and incubated for 1 hour at 37°C. The 293 cells were then added to all wells and the

2145641

plates were incubated until the serum-free control wells exhibited >95% cytopathic effect. The titer was calculated as the product of the reciprocal of the initial dilution times the reciprocal of the dilution in the last well showing >95% cytopathic effect.

5 Bronchoalveolar lavage and nasal brushings for cytology

Bronchoalveolar lavage (BAL) was performed by cannulating the trachea with a silastic catheter and injecting 5 ml of PBS. Gentle suction was applied to recover the fluid. The BAL sample was spun at 5000 rpm for 5 min. and cells were resuspended in 293 media at a concentration of 10^6 cells/ml. Cells were obtained from the monkey's nasal epithelium by gently rubbing the nasal mucosa for about 3 sec. with a cytobrush. The resulting cells were dislodged from the brushes into 2 ml of PBS. Forty microliters of the cell suspension were cytocentrifuged onto slides and stained with Wright's stain. Samples were examined by light microscopy.

15

Histology of lung sections and nasal biopsies

The right lung of each cotton rat was removed, inflated with 4% formaldehyde, and embedded in paraffin for sectioning. Nasal biopsies from the monkeys were also fixed with 4% formaldehyde. Histologic sections were stained with hematoxylin and eosin (H&E).

20 Sections were reviewed by at least one of the study personnel and by a pathologist who was unaware of the treatment each rat received.

Immunocytochemistry

Pieces of lung and trachea of the cotton rats and nasal biopsies were frozen in liquid nitrogen on O.C.T. compound. Cryosections and paraffin sections of the specimens were used for immunofluorescence microscopy. Cytospin slides of nasal brushings were prepared on gelatin coated slides and fixed with paraformaldehyde. The tissue was permeabilized with Triton X-100, then a pool of monoclonal antibodies to CFTR (M13-1, M1-4) (Denning, G.M. et al. (1992) *J. Clin. Invest.* 89:339-349) was added and incubated for 12 hours. The primary antibody was removed and an anti-mouse biotinylated antibody (Biomed, Foster City, CA) was added. After removal of the secondary antibody, streptavidin FITC (Biomed, Foster City, Ca) was added and the slides were observed under a laser scanning confocal microscope. Both control animal samples and non-immune IgG stained samples were used as controls.

35

PCR

PCR was performed on pieces of small bowel, brain, heart, kidney, liver, ovaries, and spleen from cotton rats. Approximately 1 g of the rat organs was mechanically ground and mixed with 50 μ l sterile water, boiled for 5 min., and centrifuged. A 5 μ l aliquot of the

supernatant was removed for further analysis. Monkey nasal brushings suspensions were also used for PCR.

Nested PCR primer sets were designed to selectively amplify Ad2/CFTR-1 DNA over endogenous CFTR by placing one primer from each set in the adenovirus sequence and the other primer in the CFTR sequence. The first primer set amplifies a 723 bp fragment and is shown below:

Ad2 5' ACT CTT GAG TGC CAG CGA GTA GAG TTT TCT CCT CCG 3' (SEQ ID NO:4)

CFTR 5' GCA AAG GAG CGA TCC ACA CGA AAT GTG CC 3' (SEQ ID NO:5)

The nested primer set amplifies a 506 bp fragment and is shown below:

Ad2 5' CTC CTC CGA GCC GCT CCG AGC TAG 3' (SEQ ID NO:6)

CFTR 5' CCA AAA ATG GCT GGG TGT AGG AGC AGT GTC C 3' (SEQ ID NO:7)

A PCR reaction mix containing 10mM Tris-Cl (pH 8.3), 50mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 400 µM each dNTP, 0.6 µM each primer (first set), and 2.5 units AmpliTaq (Perkin Elmer) was aliquoted into separate tubes. A 5 µl aliquot of each sample prep was then added and the mixture was overlaid with 50 µl of light mineral oil. The samples were processed on a Barnstead/Thermolyne (Dubuque, IA) thermal cycler programmed for 1 min. at 94°C, 1 min. at 65°C, and 2 min. at 72°C for 40 cycles. Post-run dwell was for 7 min. at 72°C. A 5 µl aliquot was removed and added to a second PCR reaction using the nested set of primers and cycled as above. A 10 µl aliquot of the final amplification reaction was analyzed on a 1% agarose gel and visualized with ethidium bromide.

To determine the sensitivity of this procedure, a PCR mix containing control rat liver supernatant was aliquoted into several tubes and spiked with dilutions of Ad2/CFTR-1. Following the amplification protocols described above, it was determined that the nested PCR procedure could detect as little as 50 pfu of viral DNA.

RT-PCR

RT-PCR was used to detect vector-generated mRNA in cotton rat lung tissue and samples from nasal brushings from monkeys. A 200 µl aliquot of guanidine isothiocyanate solution (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol) was added to a frozen section of each lung and pellet from nasal brushings and the tissue was mechanically ground. Total RNA was isolated utilizing a single-step method (Chomczynski, P. and Sacchi, N. et al. (1987) *Analytical Biochemistry* 162:156-159; Hanson, C.A. et al. (1990) *Am. J. Pathol.* 137:1-6). The RNA was incubated with 1 unit RQ1 RNase-free DNase (Promega Corp., Madison WI) at 37°C for 20 min., denatured at 99°C for 5 min., precipitated with ammonium acetate and ethanol, and redissolved in 4 µl diethylpyrocarbonate treated water containing 20 units RNase Block 1 (Stratagene, La Jolla CA). A 2 µl aliquot of the purified RNA was reverse transcribed using

72648-7

-39-

the GeneAmp^{*} RNA PCR kit (Perkin Elmer Cetus) and the downstream primer from the first primer set described in the previous section. Reverse transcriptase was omitted from the reaction with the remaining 2 µl of the purified RNA prep, as a control in which preparations (both +/- RT) were then amplified using nested primer sets and the PCR protocols described above. A 10 µl aliquot of the final amplification reaction was analyzed on a 1% agarose gel and visualized with ethidium bromide.

Southern analysis.

To verify the identity of the PCR products, Southern analysis was performed. The DNA was transferred to a nylon membrane as described (Sambrook et al., *supra*). A fragment of CFTR cDNA (amino acids #1-525) was labeled with [³²P]-dCTP (ICN Biomedicals, Inc. Irvine CA) using an oligolabeling kit (Pharmacia, Piscataway, NJ) and purified over a NICK column (Pharmacia Piscataway, NJ) for use as a hybridization probe. The labeled probe was denatured, cooled, and incubated with the prehybridized filter for 15 hours at 42°C. The hybridized filter was then exposed to film (Kodak XAR-5) for 10 min.

Culture of Ad2/CFTR-1

Viral cultures were performed on the permissive 293 cell line. For culture of virus from lung tissue, 1 g of lung was frozen/thawed 3-6 times and then mechanically disrupted in 200 µl of 293 media. For culture of BAL and monkey nasal brushings, the cell suspension was spun for 5 min and the supernatant was collected. Fifty µl of the supernatant was added in duplicate to 293 cells grown in 96 well plates at 50% confluence. The 293 cells were incubated for 72 hr at 37°C, then fixed with a mixture of equal parts of methanol and acetone for 10 min. and incubated with FITC-labeled anti-adenovirus monoclonal antibodies (Chemicon, Light Diagnostics, Temecuca, CA) for 30 min. Positive nuclear immunofluorescence was interpreted as positive culture. The sensitivity of the assay was evaluated by adding dilutions of Ad2/CFTR-1 to 50 µl of the lung homogenate from one of the control rats. Viral replication was detected when as little as 1 pfu was added.

RESULTS

Efficacy of Ad2/CFTR-1 in the lungs of cotton rats.

To test the ability of Ad2/CFTR-1 to transfer CFTR cDNA to the intrapulmonary airway epithelium, several studies were performed. 4 x 10⁶ pfu - IU of Ad2/CFTR-1 in 100 µl was administered to seven cotton rats; three control rats received 100 µl of TBS (the vehicle for the virus). The rats were sacrificed 4, 10 or 14 days later. To detect viral transcripts encoding CFTR, reverse transcriptase was used to prepare cDNA from lung homogenates. The cDNA was amplified with PCR using primers that span adenovirus and CFTR-encoded

* Trade-mark

sequences. Thus, the procedure did not detect endogenous rat CFTR. Figure 16 shows that the lungs of animals which received Ad2/CFTR-1 were positive for virally-encoded CFTR mRNA. The lungs of all control rats were negative.

To detect the protein, lung sections were immunostained with antibodies specific to CFTR. CFTR was detected at the apical membrane of bronchial epithelium from all rats exposed to Ad2/CFTR-1, but not from control rats. The location of recombinant CFTR at the apical membrane is consistent with the location of endogenous CFTR in human airway epithelium. Recombinant CFTR was detected above background levels because endogenous levels of CFTR in airway epithelia are very low and thus, difficult to detect by immunocytochemistry (Trapnell, B. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:6565-6569; Denning, G.M. et al. (1992) *J. Cell Biol.* 118:551-59).

These results show that Ad2/CFTR-1 directs the expression of CFTR mRNA in the lung of the cotton rat and CFTR protein in the intrapulmonary airways.

15 Safety of Ad2/CFTR-1 in cotton rats.

Because the E1 region of Ad2 is deleted in the Ad2/CFTR-1 virus, the vector was expected to be replication-impaired (Berkner, K.L. (1988) *BioTechniques* 6:616-629) and that it would be unable to shut off host cell protein synthesis (Basuss, L.E. et al. (1989) *J. Virol.* 50:202-212). Previous *in vitro* studies have suggested that this is the case in a variety of cells including primary cultures of human airway epithelial cells (Rich, D.P. et al. (1993) *Human Gene Therapy* 4:461-476). However, it is important to confirm this *in vivo* in the cotton rat, which is the most permissive animal model for human adenovirus infection (Ginsberg, H.S. et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:3823-3827; Prince, G.A. et al. (1993) *J. Virol* 67:101-111). Although dose of virus of 4.1×10^{10} pfus per kg was used, none of the rats died. More importantly, extracts from lung homogenates from each of the cotton rats were cultured in the permissive 293 cell line. With this assay 1 pfu of recombinant virus was detected in lung homogenate. However, virus was not detected by culture in the lungs of any of the treated animals. Thus, the virus did not appear to replicate *in vivo*.

It is also possible that administration of Ad2/CFTR-1 could cause an inflammatory response, either due to a direct effect of the virus or as a result of administration of viral particles. Several studies were performed to test this possibility. None of the rats had a change in the total or differential white blood cell count, suggesting that there was no major systemic inflammatory response. To assess the pulmonary inflammatory response more directly, bronchoalveolar lavage was performed on each of the rats (Figures 17A and 17B). Figure 17A shows that there was no change in the total number of cells recovered from the lavage or in the differential cell count.

Sections of the lung stained by H&E were also prepared. There was no evidence of viral inclusions or any other changes characteristic of adenoviral infection (Prince, G.A. et al. (1993) *J. Virol.* 67:101-111). When coded lung sections were evaluated by a skilled reader

2145641

- 41 -

who was unaware of which sections were treated, she was unable to distinguish between sections from the treated and untreated lungs.

It seemed possible that the recombinant adenovirus could escape from the lung into other tissues. To test for this possibility, other organs from the rats were evaluated using nested PCR to detect viral DNA. All organs tested from infected rats were negative, with the exception of small bowel which was positive in 3 of 7 rats. Figure 18 shows the results of 2 infected rats and one control rat sacrificed on day 4 after infection. The organ homogenates from the infected rats sacrificed were negative for Ad2/CFTR-1 with the exception of the small bowel. Organ homogenates from control rats sacrificed on day 4 after infection were negative for Ad2/CFTR-1. The presence of viral DNA in the small bowel suggests that the rats may have swallowed some of the virus at the time of instillation or, alternatively, the normal airway clearance mechanisms may have resulted in deposition of viral DNA in the gastrointestinal tract. Despite the presence of viral DNA in homogenates of small intestine, none of the rats developed diarrhea. This result suggests that if the virus expressed CFTR in the intestinal epithelium, there was no obvious adverse consequence.

Repeat administration of Ad2/CFTR-1 to cotton rats

Because adenovirus DNA integration into chromosomal DNA is not necessary for gene expression and only occurs at very low frequency, expression following any given treatment was anticipated to be finite and that repeated administration of recombinant adenovirus would be required for treatment of CF airway disease. Therefore, the effect of repeated administration of Ad2/CFTR-1 cotton rats was examined. Twelve cotton rats received 50 μ l of Ad2/CFTR-1. Two weeks later, 9 of the rats received a second dose of 50 μ l of Ad2/CFTR-1 and 3 rats received 50 μ l of TBS. Rats were sacrificed on day 3, 7, or 14 after virus administration. At the time of the second vector administration all cotton rats had an increased antibody titer to adenovirus.

After the second intrapulmonary administration of virus, none of the rats died. Moreover, the results of studies assessing safety and efficacy were similar to results obtained in animals receiving adenovirus for the first time. Viral cultures of rat lung homogenates on 293 cells were negative at all time points, suggesting that there was no virus replication. There was no difference between treated and control rats in the total or differential white blood count at any of the time points. The lungs were evaluated by histologic sections stained with H&E; and found no observable differences between the control and treated rats when sections were read by us or by a blinded skilled reader. Examples of some sections are shown in Figure 19. When organs were examined for viral DNA using PCR, viral DNA was found only in the small intestine of 2 rats. Despite seropositivity of the rats at the time of the second administration, expression of CFTR (as assessed by RT-PCR and by immunocytochemistry of sections stained with CFTR antibodies) similar to that seen in animals that received a single administration was observed.

These results suggest that prior administration of Ad2/CFTR-1 and the development of an antibody response did not cause an inflammatory response in the rats nor did it prevent virus-dependent production of CFTR.

5 Evidence that Ad2/CFTR-1 expresses CFTR in primate airway epithelium

The cells lining the respiratory tract and the immune system of primates are similar to those of humans. To test the ability of Ad2/CFTR-1 to transfer CFTR to the respiratory epithelium of primates, Ad2/CFTR was applied on three occasions as described in the methods to the nasal epithelium of three Rhesus monkeys. To obtain cells from the
10 respiratory epithelium, the epithelium was brushed using a procedure similar to that used to sample the airway epithelium of humans during fiberoptic bronchoscopy.

To assess gene transfer, RT-PCR was used as described above for the cotton rats. RT-PCR was positive on cells brushed from the right nostril of all three monkeys, although it was only detectable for 18 days after virus administration. An example of the results are
15 shown in Figure 20A. The presence of a positive reaction in cells from the left nostril most likely represents some virus movement to the left side due to drainage, or possibly from the monkey moving the virus from one nostril to the other with its fingers after it recovered from anesthesia.

The specificity of the RT-PCR is shown in Figure 20B. A Southern blot with a probe to CFTR hybridized with the RT-PCR product from the monkey infected with Ad2/CFTR-1.
20 As a control, one monkey received a different virus (Ad2/ β Gal-1) which encodes β -galactosidase. When different primers were used to reverse transcribe the β -galactosidase mRNA and amplify the cDNA, the appropriate PCR product was detected. However, the PCR product did not hybridize to the CFTR probe on Southern blot. This result shows the
25 specificity of the reaction for amplification of the adenovirus-directed CFTR transcript.

The failure to detect evidence of adenovirus-encoded CFTR mRNA at 18 days or beyond suggests that the sensitivity of the RT-PCR may be low because of limited efficacy of the reverse transcriptase or because RNases may have degraded RNA after cell acquisition. Viral DNA, however, was detected by PCR in brushings from the nasal epithelium for
30 seventy days after application of the virus. This result indicates that although mRNA was not detected after 2 weeks, viral DNA was present for a prolonged period and may have been transcriptionally active.

To assess the presence of CFTR proteins directly, cells obtained by brushing were plated onto slides by cytopsin and stained with antibodies to CFTR. Figure 21 shows an
35 example of the immunocytochemistry of the brushed cells. A positive reaction is clearly evident in cells exposed to Ad2/CFTR-1. The cells were scored as positive by immunocytochemistry when evaluated by a reader uninformed to the identity of the samples. Immunocytochemistry remained positive for five to six weeks for the three monkeys, even after the second administration of Ad2/CFTR-1. On occasion, a few positive staining cells

2145641

were observed from the contralateral nostril of the monkeys. However, this was of short duration, lasting at most one week.

Sections of nasal turbinate biopsies obtained within a week after the third infection were also examined. In sections from the control monkey, little if any immunofluorescence from the surface epithelium was observed, but the submucosal glands showed significant staining of CFTR (Fig. 22). These observations are consistent with results of previous studies (Engelhardt, J.F. and Wilson, J.M. (1992) *Nature Gen.* 2:240-248.) In contrast, sections from monkeys that received Ad2/CFTR-1 revealed increased immunofluorescence at the apical membrane of the surface epithelium. The submucosal glands did not appear to have greater immunostraining than was observed under control conditions. These results indicate that Ad2/CFTR-1 can transfer the CFTR cDNA to the airway epithelium of Rhesus monkeys, even in seropositive animals (see below).

Safety of Ad2/CFTR-1 administered to monkeys

Figure 23 shows that all three treated monkeys developed antibodies against adenovirus. Antibody titers measured by ELISA rose within two weeks after the first infection. With subsequent infections the titer rose within days. The sentinel monkey had low antibody titers throughout the experiment. Tests for the presence of neutralizing antibodies were also performed. After the first administration, neutralizing antibodies were not observed, but they were detected after the second administration and during the third viral administration (Fig. 23).

To detect virus, supernatants from nasal brushings and swabs were cultured on 293 cells. All monkeys had positive cultures on day 1 and on day 3 or 4 from the infected nostril. Cultures remained positive in one of the monkeys at seven days after administration, but cultures were never positive beyond 7 days. Live virus was occasionally detected in swabs from the contra lateral nostril during the first 4 days after infection. The rapid loss of detectable virus suggests that there was not viral replication. Stools were routinely cultured, but virus was never detected in stools from any of the monkeys.

None of the monkeys developed any clinical signs of viral infection or inflammation. Visual inspection of the nasal epithelium revealed slight erythema in all three monkeys in both nostrils on the first day after infection; but similar erythema was observed in the control monkey and likely resulted from the instrumentation. There was no visible abnormalities at days 3 or 4, or on weekly inspection thereafter. Physical examination revealed no fever, lymphadenopathy, conjunctivitis, tachypnea, or tachycardia at any of the time points. No abnormalities were found in a complete blood count or sedimentation rate, nor were abnormalities observed in serum electrolytes, transaminases, or blood urea nitrogen and creatinine.

Examination of Wright-stained cells from the nasal brushings showed that neutrophils and lymphocytes accounted for less than 5% of total cells in all three monkeys.

Administration of the Ad2/CFTR-1 caused no change in the distribution or number of inflammatory cells at any of the time points following virus administration. H&E stains of the nasal turbinate biopsies specimens from the control monkey could not be differentiated from that of the experimental monkey when the specimens were reviewed by an independent pathologist. (Fig. 24)

These results demonstrate the ability of a recombinant adenovirus encoding CFTR (Ad2/CFTR-1) to express CFTR cDNA in the airway epithelium of cotton rats and monkeys during repeated administration. They also indicate that application of the virus involves little if any risk. Thus, they suggest that such a vector may be of value in expressing CFTR in the airway epithelium of humans with cystic fibrosis.

Two methods were used to show that Ad2/CFTR-1 expresses CFTR in the airway epithelium of cotton rats and primates: CFTR mRNA was detected using RT-PCR and protein was detected by immunocytochemistry. Duration of expression as assessed immunocytochemically was five to six weeks. Because very little protein is required to generate Cl^- secretion (Welsh, M.J. (1987) *Physiol. Rev.* 67:1143-1184; Trapnell, B.C. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:6565-6569; Denning, G.M. et al. (1992) *J. Cell Biol.* 118:551-559), it is likely that functional expression of CFTR persists substantially longer than the period of time during which CFTR was detected by immunocytochemistry. Support for this evidence comes from two considerations: first, it is very difficult to detect CFTR immunocytochemically in the airway epithelium, yet the expression of an apical membrane Cl^- permeability due to the presence of CFTR Cl^- channels is readily detected. The ability of a minimal amount of CFTR to have important functional effects is likely a result of the fact that a single ion channel conducts a very large number of ions ($10^6 - 10^7$ ions/sec). Thus, ion channels are not usually abundant proteins in epithelia. Second, previous work suggests that the defective electrolyte transport of CF epithelia can be corrected when only 6-10% of cells in a CF airway epithelium overexpress wild-type CFTR (Johnson, L.G. et al. (1992) *Nature Gen.* 2:21-25). Thus, correction of the biologic defect in CF patients may be possible when only a small percent of the cells express CFTR. This is also consistent with our previous studies *in vitro* showing that Ad2/CFTR-1 at relatively low multiplicities of infection generated a cAMP-stimulated Cl^- secretory response in CF epithelia (Rich, D.P. et al. (1993) *Human Gene Therapy* 4:461-476).

This study also provides the first comprehensive data on the safety of adenovirus vectors for gene transfer to airway epithelium. Several aspects of the studies are encouraging. There was no evidence of viral replication, rather infectious viral particles were rapidly cleared from both cotton rats and primates. These data, together with our previous *in vitro* studies, suggest that replication of recombinant virus in humans will likely not be a problem. The other major consideration for safety of an adenovirus vector in the treatment of CF is the possibility of an inflammatory response. The data indicate that the virus generated an antibody response in both cotton rats and monkeys. Despite this, no evidence of a

2145641

- 45 -

systemic or local inflammatory response was observed. The cells obtained by bronchoalveolar lavage and by brushing and swabs were not altered by virus application. Moreover, the histology of epithelia treated with adenovirus was indistinguishable from that of control epithelia. These data suggest that at least three sequential exposures of airway epithelium to adenovirus does not cause a detrimental inflammatory response.

These data suggest that Ad2/CFTR-1 can effectively transfer CFTR cDNA to airway epithelium and direct the expression of CFTR. They also suggest that transfer is relatively safe in animals. Thus, they suggest that Ad2/CFTR-1 may be a good vector for treating patients with CF. This was confirmed in the following example.

Example 10 - CFTR Gene Therapy in Nasal Epithelia from Human CF Subjects

EXPERIMENTAL PROCEDURES

Adenovirus vector

The recombinant adenovirus Ad2/CFTR-1 was used to deliver CFTR cDNA. The construction and preparation of Ad2/CFTR-1, and its use *in vitro* and *in vivo* in animals, has been previously described (Rich, D.P. et al. (1993) *Human Gene Therapy* 4:461-476; Zabner, J. et al. (1993) *Nature Gen.* (in press)). The DNA construct comprises a full length copy of the Ad2 genome from which the early region 1 genes (nucleotides 546 to 3497) have been replaced by cDNA for CFTR. The viral E1a promoter was used for CFTR cDNA; this is a low to moderate strength promoter. Termination/polyadenylation occurs at the site normally used by E1b and protein IX transcripts. The E3 region of the virus was conserved.

Patients

Three patients with CF were studied. Genotype was determined by IG Labs (Framingham, MA). All three patients had mild CF as defined by an NIH score > 70 (Taussig, L.M. et al. (1973) *J. Pediatr.* 82:380-390), a normal weight for height ratio, a forced expiratory volume in one second (FEV1) greater than 50% of predicted and an arterial PO₂ greater than 72. All patients were seropositive for type 2 adenovirus, and had no recent viral illnesses. Pretreatment cultures of nasal swabs, pharyngeal swabs, sputum, urine, stool, and blood leukocytes were negative for adenovirus. PCR of pretreatment nasal brushings using primers for the adenovirus E1 region were negative. Patients were evaluated at least twice by FEV1, cytology of nasal mucosa, visual inspection, and measurement of V_t before treatment. Prior to treatment, a coronal computed tomographic scan of the paranasal sinuses and a chest X-ray were obtained.

The first patient was a 21 year old woman who was diagnosed at 3 months after birth. She had pancreatic insufficiency, a positive sweat chloride test (101 mEq/l), and is homozygous for the $\Delta F508$ mutation. Her NIH score was 90 and her FEV1 was 83%

predicted. The second patient was a 36 year old man who was diagnosed at the age of 13 when he presented with symptoms of pancreatic insufficiency. A sweat chloride test revealed a chloride concentration of 70 mEq/l. He is a heterozygote with the $\Delta F508$ and G551D mutations. His NIH score was 88 and his FEV1 was 66% predicted. The third patient was a
5 50 year old woman, diagnosed at the age of 9 with a positive sweat chloride test (104 mEq/l). She has pancreatic insufficiency and insulin dependent diabetes mellitus. She is homozygous for the $\Delta F508$ mutation. Her NIH score was 73 and her FEV1 was 65% predicted.

Transepithelial voltage

10 The transepithelial electric potential difference across the nasal epithelium was measured using techniques similar to those previously described (Alton, E.W.F.W. et al (1987) *Thorax* 42:815-817; Knowles, M. et al. (1981) *N. Eng. J. Med.* 305:1489-1495). A 23 gauge subcutaneous needle connected with sterile normal saline solution to a silver/silver chloride pellet (E.W. Wright, Guilford, CT) was used as a reference electrode. The exploring
15 electrode was a size 8 rubber catheter (modified Argyle^R Foley catheter, St. Louis, MO) with one side hole at the tip. The catheter was filled with Ringer's solution containing (in mM), 135 NaCl, 2.4 KH_2PO_2 , K_2HPO_4 , 1.2 CaCl_2 , 1.2 MgCl_2 and 10 Hepes (titrated to pH 7.4 with NaOH) and was connected to a silver/silver chloride pellet. Voltage was measured with a voltmeter (Keithley Instruments Inc., Cleveland, OH) connected to a strip chart recorder
20 (Servocorder, Watanabe Instruments, Japan). Prior to the measurements, the silver/silver chloride pellets were connected in series with the Ringer's solution; the pellets were changed if the recorded V_t was greater than ± 4 mV. The rubber catheter was introduced into the nostril under telescopic guidance (Hopkins Telescope, Karl Storz, Tuttlingen West Germany) and the side hole of the catheter was placed next to the study area in the medical aspect of the
25 inferior nasal turbinate. The distance from the anterior tip of the inferior turbinate and the spatial relationship with the medial turbinate, the maxillary sinus ostium, and in one patient a small polyp, were used to locate the area of Ad2/CFTR-1 administration for measurements. Photographs and video recorder images were also used. Basal V_t was recorded until no changes in V_t were observed after slow intermittent 100 $\mu\text{l}/\text{min}$ infusion of the Ringer's
30 solution. Once a stable baseline was achieved, 200 μl of a Ringer's solution containing 100 μM amiloride (Merck and Co. Inc., West Point, PA) was instilled through the catheter and changes in V_t were recorded until no further change were observed after intermittent instillations. Finally, 200 μl Ringer's solution containing 100 μM amiloride plus 10 μM terbutaline (Geigy Pharmaceuticals, Ardsley, NY) was instilled and the changes in V_t were
35 recorded.

Measurements of basal V_t were reproducible over time: in the three treated patients, the coefficients of variation before administration of Ad2/CFTR-1 were 3.6%, 12%, and 12%. The changes induced by terbutaline were also reproducible. In 30 measurements in 9 CF patients, the terbutaline-induced changes in V_t (ΔV_t) ranged from 0 mV to +4 mV;

2145641

- 47 -

hyperpolarization of V_t was never observed. In contrast, in 7 normal subjects ΔV_t ranged from -1 mV to -5 mV; hyperpolarization was always observed.

Ad2/CFTR-1 application and cell acquisition

5 The patients were taken to the operating room and monitoring was commenced using continuous EKG and pulse oximetry recording as well as automatic intermittent blood pressure measurement. After mild sedation, the nasal mucosa was anesthetized by atomizing 0.5 ml of 5% cocaine. The mucosa in the area of the inferior turbinate was then packed with cotton pledgets previously soaked in a mixture of 2 ml of 0.1% adrenaline and 8 ml of 1% tetracaine. The pledgets remained in place for 10-40 min. Using endoscopic visualization with a television monitoring system, the applicator was introduced through the nostril and positioned on the medial aspect of the inferior turbinate, at least three centimeters from its anterior tip (Figures 25A-25I). The viral suspension was infused into the applicator through connecting catheters. The position of the applicator was monitored endoscopically to ensure that it did not move and that enough pressure was applied to prevent leakage. After the virus was in contact with the nasal epithelium for thirty minutes, the viral suspension was removed, and the applicator was withdrawn. In the third patient's right nasal cavity, the virus was applied using the modified Foley catheter used for V_t measurements. The catheter was introduced without anesthetic under endoscopic guidance until the side hole of the catheter was in contact with the area of interest in the inferior turbinate. The viral solution was infused slowly until a drop of solution was seen with the telescope. The catheter was left in place for thirty minutes and then removed.

Cells were obtained from the area of virus administration approximately 2 weeks before treatment and then at weekly intervals after treatment. The inferior turbinate was packed for 10 minutes with cotton pledgets previously soaked in 1 ml of 5% cocaine. Under endoscopic control, the area of administration was gently brushed for 5 seconds. The brushed cells were dislodged in PBS. Swabs of the nasal epithelia were collected using cotton tipped applicators without anesthesia. Cytospin slides were prepared and stained with Wright's stain. Light microscopy was used to assess the respiratory epithelial cells and inflammatory cells. For biopsies, sedatives/anesthesia was administered as described for the application procedure. After endoscopic inspection, and identification of the site to be biopsied, the submucosa was injected with 1% xylocaine, with 1/100,000 epinephrine. The area of virus application on the inferior turbinate was removed. The specimen was fixed in 4% formaldehyde and stained.

35

RESULTS

On day one after Ad2/CFTR-1 administration and at all subsequent time points, Ad2/CFTR-1 from the nasal epithelium, pharynx, blood, urine, or stool could not be cultured. As a control for the sensitivity of the culture assay, samples were routinely spiked with 10

and 100 IU Ad2/CFTR-1. In every case, the spiked samples were positive, indicating that, at a minimum, 10 IU of Ad2/CFTR should have been detected. No evidence of a systemic response as assessed by history, physical examination, serum chemistries or cell counts, chest and sinus X-rays, pulmonary function tests, or arterial blood gases performed before and after Ad2/CFTR-1 administration. An increase in antibodies to adenovirus was not detectable by ELISA or by neutralization for 35 days after treatment.

Three to four hours after Ad2/CFTR-1 administration, at the time that local anesthesia and localized vasoconstriction abated, all patients began to complain of nasal congestion and in one case, mild rhinorrhea. These were isolated symptoms that diminished by 18 hours and resolved by 28 to 42 hours. Inspection of the nasal mucosa showed mild to moderate erythema, edema, and exudate (Figures 25A-25C). These physical findings followed a time course similar to the symptoms. The physical findings were not limited to the site of virus application, even though preliminary studies using the applicator showed that marker methylene blue was limited to the area of application. In two additional patients with CF, the identical anesthesia and application procedure were used, but saline was applied instead of virus, yet the same symptoms and physical findings were observed in these patients (Figures 25G-25I). Moreover, the local anesthesia and vasoconstriction generated similar changes even when the applicator was not used, suggesting that the anesthesia/vasoconstriction caused some, if not all the injury. Twenty-four hours after the application procedure, analysis of cells removed from nasal swabs revealed an equivalent increase in the percent neutrophils in patients treated with Ad2/CFTR-1 or with saline. One week after application, the neutrophilia had resolved in both groups. Respiratory epithelial cells obtained by nasal brushing appeared normal at one week and at subsequent time points, and showed no evidence of inclusion bodies. To further evaluate the mucosa, the epithelium was biopsied on day three in the first patient and day one in the second patient. Independent evaluation by two pathologists not otherwise associated with the study suggested changes consistent with mild trauma and possible ischemia (probably secondary to the anesthetic/vasoconstrictors used before virus administration), but there were no abnormalities suggestive of virus-mediated damage.

Because the application procedure produced some mild injury in the first two patients, the method of administration was altered in the third patient. The method used did not require the use of local anesthesia or vasoconstriction and which was thus less likely to cause injury, but which was also less certain in its ability to constrain Ad2/CFTR-1 in a precisely defined area. On the right side, Ad2/CFTR-1 was administered as in the first two patients, and on the left side, the virus was administered without anesthesia or the applicator, instead using a small Foley catheter to apply and maintain Ad2/CFTR-1 in a relatively defined area by surface tension (Figure 25E). On the right side, the symptoms and physical findings were the same as those observed in the first two patients. By contrast, on the left side there were no symptoms and on inspection the nasal mucosa appeared normal (Figures 25D-25F). Nasal

2145641

swabs obtained from the right side showed neutrophilia similar to that observed in the first two patients. In contrast, the left side which had no anesthesia and minimal manipulation, did not develop neutrophilia. Biopsy of the left side on day 3 after administration (Figure 26), showed morphology consistent with CF-- a thickened basement membrane and occasional polymorphonuclear cells in the submucosa-- but no abnormalities that could be attributed to the adenovirus vector.

The first patient developed symptoms of a sore throat and increased cough that began three weeks after treatment and persisted for two days. Six weeks after treatment she developed an exacerbation of her bronchitis/bronchiectasis and hemoptysis that required hospitalization. The second patient had a transient episode of minimal hemoptysis three weeks after treatment; it was not accompanied by any other symptoms before or after the episode. The third patient has an exacerbation of bronchitis three weeks after treatment for which she was given oral antibiotics. Based on each patient's pretreatment clinical history, evaluation of the episodes, and viral cultures, no evidence could be discerned that linked these episodes to administration of Ad2/CFTR-1. Rather the episodes appeared consistent with the normal course of disease in each individual.

The loss of CFTR Cl^- channel function causes abnormal ion transport across affected epithelia, which in turn contributes to the pathogenesis of CF-associated airway disease (Boat, T.F. et al. in *The Metabolic Basis of Inherited Diseases* (Scriver, C.R. et al. eds., McGraw-Hill, New York (1989); Quinton, P.M. (1990) *FASEB J.* 4:2709-2717). In airway epithelia, ion transport is dominated by two electrically conductive processes: amiloride-sensitive absorption of Na^+ from the mucosal to the submucosal surface and cAMP-stimulated Cl^- secretion in the opposite direction. (Quinton, P.M. (1990) *FASEB J.* 4:2709-2717; Welsh, M.J. (1987) *Physiol. Rev.* 67:1143-1184). These two transport processes can be assessed noninvasively by measuring the voltage across the nasal epithelium (V_t) *in vivo* (Knowles, M. et al (1981) *N. Eng. J. Med.* 305:1489-1495; Alton, E.W.F.W. et al.(1987) *Thorax* 42:815-817). Figure 27 shows an example from a normal subject. Under basal conditions, V_t was electrically negative (lumen referenced to the submucosal surface). Perfusion of amiloride (100 μM) onto the mucosal surface inhibited V_t by blocking apical Na^+ channels (Knowles, M. et al (1981) *N. Eng. J. Med.* 305:1489-1495; Quinton, P.M. (1990) *FASEB J.* 4:2709-2717; Welsh, M.J. (1992) *Neuron* 8:821-829). Subsequent perfusion of terbutaline (10 μM) a β -adrenergic agonist, hyperpolarized V_t by increasing cellular levels of cAMP, opening CFTR Cl^- channels, and stimulating chloride secretion (Quinton, P.M. (1990) *FASEB J.* 4:2709-2717; Welsh, M.J. et al. (1992) *Neuron* 8:821-829). Figure 28A shows results from seven normal subjects: basal V_t was $-10.5 \pm 1.0\text{mV}$, and in the presence of amiloride, terbutaline hyperpolarized V_t by $-2.3 \pm 0.5\text{mV}$.

In patients with CF, V_t was more electrically negative than in normal subjects (Figure 28B), as has been previously reported (Knowles, M. et al. (1981) *N. Eng. J. Med.* 305:1489-1495). Basal V_t was $-37.0 \pm 2.4\text{mV}$, much more negative than values in normal subjects ($P <$

0.001). (Note the difference in scale in Figure 28A and Figure 28B). Amiloride inhibited V_t , as it did in normal subjects. However, V_t failed to hyperpolarize when terbutaline was perfused onto the epithelium in the presence of amiloride. Instead, V_t either did not change or became less negative: on average V_t depolarized by $+1.8 \pm 0.6$ mV, a result very different from that observed in normal subjects. ($P < 0.001$).

After Ad2/CFTR-1 was applied, basal V_t became less negative in all three CF patients: Figure 29A shows an example from the third patient before (Figure 29A) and after (Figure 29B) treatment and Figures 30A, 30C, and 30E show the time course of changes in basal V_t for all three patients. The decrease in basal V_t suggests that application of Ad2/CFTR-1 corrected the CF electrolyte transport defect in nasal epithelium of all three patients. Additional evidence came from an examination of the response to terbutaline. Figure 30B shows that in contrast to the response before Ad2/CFTR-1 was applied, after virus replication, in the presence of amiloride, terbutaline stimulated V_t . Figures 30B, 30D, and 30F show the time course of the response. These data indicate that Ad2/CFTR-1 corrected the CF defect in Cl^- transport. Correction of the Cl^- transport defect cannot be attributed to the anesthesia/application procedure because it did not occur in patients treated with saline instead of Ad2/CFTR-1 (Figure 31). Moreover, the effects of the anesthesia were generalized on the nasal mucosa, but basal V_t decreased only in the area of virus administration. Finally, similar changes were observed in the left nasal mucosa of the third patient (Figures 30E and 30F), which had no symptomatic or physical response after the modified application procedure.

Unsuccessful attempts were made to detect CFTR transcripts by reverse transcriptase-PCR and by immunocytochemistry in cells from nasal brushings and biopsies. Although similar studies in animals have been successful (Zabner, J. et al. (1993) *Nature Gen.* (in press)), those studies used much higher doses of Ad2/CFTR-1. The lack of success in the present case likely reflects the small amount of available tissue, the low MOI, the fact that only a fraction of cells may have been corrected, and the fact that Ad2/CFTR-1 contains a low to moderate strength promoter (E1a) which produces much less mRNA and protein than comparable constructs using a much stronger CMV promoter (unpublished observation). The E1a promoter was chosen because CFTR normally expressed at very low levels in airway epithelial cells (Trapnell, B.C. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:6565-6569). It is also difficult to detect CFTR protein and mRNA in normal human airway epithelia, although function is readily detected because a single ion channel can conduct a very large number of ions per second and thus efficiently support Cl^- transport.

With time, the electrical changes that indicate correction of the CF defect reverted toward pretreatment values. However, the basal V_t appeared to revert more slowly than did the change in V_t produced by terbutaline. The significance of this difference is unknown, but it may reflect the relative sensitivity of the two measurements to expression of normal CFTR. In any case, this study was not designed to test the duration of correction because the treated

2145641

- 51 -

area was removed by biopsy on one side and the nasal mucosa on the other side was brushed to obtain cells for analysis at 7 to 10 days after virus administration, and then at approximately weekly intervals. Brushing the mucosa removes cells, disrupts the epithelium, and reduces basal V_t to zero for at least two days afterwards, thus preventing an accurate assessment of duration of the effect of Ad2/CFTR-1.

Efficacy of adenovirus-mediated gene transfer.

The major conclusion of this study is that *in vivo* application of a recombinant adenovirus encoding CFTR can correct the defect in airway epithelial Cl^- transport that is characteristic of CF epithelia.

Complementation of the Cl^- channel defect in human nasal epithelium could be measured as a change in basal voltage and as a change in the response to cAMP agonists. Although the protocol was not designed to establish duration, changes in these parameters were detected for at least three weeks. These results represent the first report that administration of a recombinant adenovirus to humans can correct a genetic lesion as measured by a functional assay. This study contrasts with most earlier attempts at gene transfer to humans, in that a recombinant viral vector was administered directly to humans, rather than using a *in vitro* protocol involving removal of cells from the patient, transduction of the cells in culture, followed by reintroduction of the cells into the patient.

Evidence that the CF Cl^- transport defect was corrected at all three doses of virus, corresponding to 1, 3, and 25 MOI, was obtained. This result is consistent with earlier studies showing that similar MOIs reversed the CF fluid and electrolyte transport defects in primary cultures of CF airway cells grown as epithelia on permeable filter supports (Rich, D.P. et al. (1993) *Human Gene Therapy* 4:461-476 and Zabner et al. submitted for publication): at an MOI of less than 1, cAMP-stimulated Cl^- secretion was partially restored, and after treatment with 1 MOI Ad2/CFTR-1 cAMP agonists stimulated fluid secretion that was within the range observed in epithelia from normal subjects. At an MOI of 1, a related adenovirus vector produced β -galactosidase activity in 20% of infected epithelial cells as assessed by fluorescence-activated cell analysis (Zabner et al. submitted for publication). Such data would imply that pharmacologic dose of adenovirus in CF airways might correspond to an MOI of one. If it is estimated that there are 2×10^6 cells/cm² in the airway (Mariassy, A.T. in *Comparative Biology of the Normal Lung* (CRC Press, Boca Raton 1992), and that the airways from the trachea to the respiratory bronchioles have a surface area of 1400 cm² (Weibel, E.R. *Morphometry of the Human Lung* (Springer Verlag, Heidelberg, 1963) then there would be approximately 3×10^9 potential target cells. Assuming a particle to IU ratio of 100, this would correspond to approximately 3×10^{11} particles of adenovirus with a mass of approximately 75 μ g. While obviously only a crude estimate, such information is useful in designing animal experiments to establish the likely safety profile of a human dose.

It is possible that an efficacious MOI of recombinant adenovirus could be less than the lowest MOI tested here. Some evidence suggests that not all cells in an epithelial monolayer need to express CFTR to correct the CF electrolyte transport defects. Mixing experiments showed that when perhaps 5-10% of cells overexpress CFTR, the monolayer exhibits wild-type electrical properties (Johnson, L.G. et al. (1992) *Nature Gen.* 2:21-25). Studies using liposomes to express CFTR in mice bearing a disrupted CFTR gene also suggest that only a small proportion of cells need to be corrected (Hyde, S.C. et al. (1993) *Nature* 362:250-255). The results referred to above using airway epithelial monolayers and multiplicities of Ad2/CFTR-1 as low as 0.1 showed measurable changes in Cl^- secretion (Rich, D.P. et al. (1993) *Human Gene Therapy* 4:461-476 and Zabner et al. submitted for publication).

Given the very high sensitivity of electrolyte transport assays (which result because a single Cl^- channel is capable of transporting large numbers of ions/sec) and the low activity of the E1a promoter used to transcribe CFTR, the inability to detect CFTR protein and CFTR mRNA are perhaps not surprising. Although CFTR mRNA could not be detected by reverse transcriptase-PCR, Ad2/CFTR-1 DNA could be detected in the samples by standard PCR, demonstrating the presence of input DNA and suggesting that the reverse transcriptase reaction may have been suboptimal. This could have occurred because of factors in the tissue that inhibit the reverse transcriptase. Although there is little doubt that the changes in electrolyte transport measured here result from expression of CFTR, it remains to be seen whether this will lead to measurable clinical changes in lung function.

Safety considerations.

Application of the adenovirus vector to the nasal epithelium in these three patients was well-tolerated. Although mild inflammation was observed in the nasal epithelium of all three patients following administration of Ad2/CFTR-1, similar changes were observed in two volunteers who underwent a sham procedure using saline rather than the viral vector. Clearly a combination of anesthetic- and procedure-related trauma resulted in the changes in the nasal mucosa. There is insufficient evidence to conclude that no inflammation results from virus administration. However, using a modified administration of the highest MOI of virus tested (25 MOI) in one patient, no inflammation was observed under conditions that resulted in evidence of biophysical efficacy that lasted until the area was removed by biopsy at three days.

There was no evidence of replication of Ad2/CFTR-1. Earlier studies had established that replication of Ad2/CFTR-1 in tissue culture and experimental animals is severely impaired (Rich, D.P. et al. (1993) *Human Gene Therapy* 4:461-476; Zabner, J. et al. (1993) *Nature Gen.* (in press)). Replication only occurs in cells that supply the missing early proteins of the E1 region of adenovirus, such as 293 cells, or under conditions where the E1 region is provided by coinfection with or recombination with an E1-containing adenovirus

(Graham, F.L. and Prevec, L. Vaccines: New Approaches to Immunological Problems (R.W. Ellis, ed., Boston, Butterworth-Heinemann, 1992); Berkner, K.L. (1988) *Biotechniques* 6:616-629). The patients studied here were seropositive for adenovirus types 2 and 5 prior to the study were negative for adenovirus upon culture of nasal swabs prior to administration of Ad2/CFTR-1, and were shown by PCR methods to lack endogenous E1 DNA sequences such as have been reported in some human subjects (Matsuse T. et al. (1992) *Am. Rev. Respir. Dis.* 146:177-184).

Example 11 - Construction and Packaging of Pseudo Adenoviral Vector (PAV)

10 With reference to Figure 32, the PAV construct was made by inserting the Ad2 packaging signal and E1 enhancer region (0-358 nt) in Bluescript II SK- (Stratagene, LaJolla, CA). A variation of this vector, known as PAV II was constructed similarly, except the Ad2 packaging signal and E1 enhancer region contained 0-380 nt. The addition of nucleotides at the 5' end results in larger PAVs, which may be more efficiently packaged, yet would include
15 more adenoviral sequences and therefore could potentially be more immunogenic or more capable of replicating.

To allow ease of manipulation for either the insertion of gene coding regions or complete excision and use in transfections for the purpose of generating infectious particles, a complementary plasmid was also built in pBluescript SKII-. This complementary plasmid
20 contains the Ad2 major late promoter (MLP) and tripartite leader (TPL) DNA and an SV40 T-antigen nuclear localization signal (NLS) and polyadenylation signal (SVpA). As can be seen in Figure 32, this plasmid contains a convenient restriction site for the insertion of genes of interest between the MLP/TPL and SV40 poly A. This construct is engineered such that the entire cassette may be excised and inserted into the former PAV I or PAV II construct.

25 Generation of PAV infectious particles was performed by excision of PAV from the plasmid with the Apa I and Sac II restriction endonucleases and co-transfection into 293 cells (an Ela/Elb expressing cell line) (Graham, F.L. et al, (1977) *J. Gen Virol* 36:59-74) with either wild-type Ad2, or packaging/replication deficient helper virus. Purification of PAV from helper can be accompanied by CsCl gradient isolation as PAV viral particles will be of a
30 lower density and will band at a higher position in the gradient.

For gene therapy, it is desirable to generate significant quantities of PAV virion free from contaminating helper virus. The primary advantage of PAV over standard adenoviral vectors is the ability to package large DNA inserts into virion (up to about 36 kb). However, PAV requires a helper virus for replication and packaging and this helper virus will be the
35 predominant species in any PAV preparation. To increase the proportion of PAV in viral preparation several approaches can be employed. For example, one can use a helper virus which is partially defective for packaging into virions (either by virtue of mutations in the packaging sequences (Grable, M. and Hearing P. (1992) *J. Virol.* 66: 723-731)) or by virtue of its size -viruses with genome sizes greater than approximately 37.5 kb package

inefficiently. In mixed infections with packaging defective virus, PAV would be expected to be represented at higher levels in the virus mixture than would occur with non-packaging defective helper viruses.

Another approach is to make the helper virus dependent upon PAV for its own replication. This may most easily be accomplished by deleting an essential gene from the helper virus (e.g. IX or a terminal protein) and placing that gene in the PAV vector. In this way neither PAV nor the helper virus is capable of independent replication - PAV and the helper virus are therefore co-dependent. This should result in higher PAV representation in the resulting virus preparation.

A third approach is to develop a novel packaging cell line, which is capable of generating significant quantities of PAV virion free from contaminating helper virus. A novel protein IX, (pIX) packaging system has been developed. This system exploits several documented features of adenovirus molecular biology. The first is that adenoviral defective particles are known to comprise up to 30% or more of standard wild-type adenoviral preparations. These defective or incomplete particles are stable and contain 15-95% of the adenoviral genome, typically 15-30%. Packaging of a PAV genome (15-30% of wild-type genome) should package comparably. Secondly, stable packaging of full-length Ad genome but not genomes <95% required the presence of the adenoviral gene designated pIX.

The novel packaging system is based on the generation of an Ad protein pIX expressing 293 cell line. In addition, an adenoviral helper virus engineered such that the E1 region is deleted but enough exogenous material is inserted to equal or slightly exceed the full length 36 kb size. Both of these two constructs would be introduced into the 293/pIX cell line as purified DNA. In the presence of pIX, yields of both predicted progeny viruses as seen in current PAV/Ad2 production experiments can be obtained. Virus containing lysates from these cells can then be titered independently (for the marker gene activity specific to either vector) and used to infect standard 293 (lacking pIX) at a multiplicity of infection of 1 relative to PAV. Since research with this line as well as from incomplete or defective particle research indicates that full length genomes have a competitive packaging advantage, it is expected that infection with an MOI of 1 relative to PAV will necessarily equate to an effective MOI for helper of greater than 1. All cells will presumably contain both PAV (at least 1) and helper (greater than 1). Replication and viral capsid production in this cell should occur normally but only PAV genomes should be packaged. Harvesting these 293/pIX cultures is expected to yield essentially helper-free PAV.

Example 12 - Construction of Ad2-E4/ORF 6

Ad2-E4/ORF6 (Figure 33 shows the plasmid construction of Ad2-E4/ORF6) which is an adenovirus 2 based vector deleted for all Ad2 sequences between nucleotides 32815 and 35577. This deletion removes all open reading frames of E4 but leaves the E4 promoter and first 32-37 nucleotides of the E4 mRNA intact. In place of the deleted sequences, a DNA

2145641

fragment encoding ORF6 (Ad2 nucleotides 34082-33178) which was derived by polymerase chain reaction of Ad2 DNA with ORF6 specific DNA primers (Genzyme oligo. # 2371 - CGGATCCTTTATTATAGGGGAAGTCCACGCCTAC (SEQ. ID NO:8) and oligo. #2372 - CGGGATCCATCGATGAAATATGACTACGTCCG (SEQ. ID NO:9) were inserted). Additional sequences supplied by the oligonucleotides included a cloning site at the 5' and 3' ends of the PCR fragment (Clal and BamHI respectively) and a polyadenylation sequence at the 3' end to ensure correct polyadenylation of the ORF6 mRNA. As illustrated in Figure 33, the PCR fragment was first ligated to a DNA fragment including the inverted terminal repeat (ITR) and E4 promoter region of Ad2 (Ad2 nucleotides 35937-35577) and cloned in the bacterial plasmid pBluescript (Stratagene) to create plasmid ORF6. After sequencing to verify the integrity of the ORF6 reading frame, the fragment encompassing the ITR and ORF6 was subcloned into a second plasmid, pAd Δ E4, which contains the 3' end of Ad2 from a Sac I site to the 3' ITR (Ad2 nucleotides 28562-35937) and is deleted for all E4 sequences (promoter to poly A site Ad2 positions 32815-35641) using flanking restriction sites. In this second plasmid, virus expressing only E4 ORF6, pAdORF6 was cut with restriction enzyme PacI and ligated to Ad2 DNA digested with PacI. This PacI site corresponds to Ad2 nucleotide 28612. 293 cells were transfected with the ligation and the resulting virus was subjected to restriction analysis to verify that the Ad2 E4 region had been substituted with the corresponding region of pAdORF6 and that the only remaining E4 open reading frame was ORF6.

A cell line could in theory be established that would fully complement E4 functions deleted from a recombinant virus. The problem with this approach is that E4 functions in the regulation of host cell protein synthesis and is therefore toxic to cells. The present recombinant adenoviruses are deleted for the E1 region and must be grown in 293 cells which complement E1 functions. The E4 promoter is activated by the Ela gene product, and therefore to prevent inadvertent toxic expression of E4 transcription of E4 must be tightly regulated. The requirements of such a promoter or transactivating system is that in the uninduced state expression must be low enough to avoid toxicity to the host cell, but in the induced state must be sufficiently activated to make enough E4 gene product to complement the E4 deleted virus during virus production.

Example 13

An adenoviral vector is prepared as described in Example 7 while substituting the phosphoglycerate kinase (PGK) promoter for the Ela promoter.

Example 14

An adenoviral vector is prepared as described in Example 11 while substituting the PGK promoter for the Ad2 major late promoter (MLP).

Example 15: Generation of Ad2-ORF6/PGK-CFTR

This protocol uses a second generation adenovirus vector named Ad2-ORF6/PGK-CFTR. This virus lacks E1 and in its place contains a modified transcription unit with the PGK promoter and a poly A addition site flanking the CFTR cDNA. The PGK promoter is of only moderate strength but is long lasting and not subject to shut off. The E4 region of the vector has also been modified in that the whole coding sequence has been removed and replaced by ORF6, the only E4 gene essential for growth of Ad in tissue culture. This has the effect of generating a genome of 101% the size of wild type Ad2.

The DNA construct comprises a full length copy of the Ad2 genome from which the early region 1 (E1) genes (present at the 5' end of the viral genome) have been deleted and replaced by an expression cassette encoding CFTR. The expression cassette includes the promoter for phosphoglycerate kinase (PGK) and a polyadenylation (poly A) addition signal from the bovine growth hormone gene (BGH). In addition, the E4 region of Ad2 has been deleted and replaced with only open reading frame 6 (ORF6) of the Ad2 E4 region. The adenovirus vector is referred to as AD2-ORF6/PGK-CFTR and is illustrated schematically in Figure 34. The entire wild-type Ad2 genome has been previously sequenced (Roberts, R.J., (1986) In Adenovirus DNA, W. Oberfler, editor, Martinus Nihoff Publishing, Boston) and the existing numbering system has been adopted here when referring to the wild type genome. Ad2 genomic regions flanking E1 and E4 deletions, and insertions into the genome are being completely sequenced.

The Ad2-ORF6/PGK-CFTR construct differs from the one used in our earlier protocol (Ad2/CFTR-1) in that the latter utilized the endogenous E1a promoter, had no poly A addition signal directly downstream of CFTR and retained an intact E4 region. The properties of Ad2/CFTR-1 in tissue culture and in animal studies have been reported (Rich et al., (1993) *Human Gene Therapy* 4:461-467; and Zabner et al. (1993) *Nature Genetics* (in Press)).

At the 5' end of the genome, nucleotides 357 to 3328 of Ad2 have been deleted and replaced with (in order 5' to 3') 22 nucleotides of linker, 534 nucleotides of the PGK promoter, 86 nucleotides of linker, nucleotides 123-4622 of the published CFTR sequence (Riordan et al. (1989) *Science* 245:1066-1073), 21 nucleotides of linker, and a 32 nucleotide synthetic BGH poly A addition signal followed by a final 11 nucleotides of linker. The topology of the 5' end of the recombinant molecule is illustrated in Figure 34.

At the 3' end of the genome of Ad2-ORF6/PGK-CFTR, Ad2 sequences between nucleotides 32815 and 35577 have been deleted to remove all open reading frames of E4 but retain the E4 promoter, the E4 cap sites and first 32-37 nucleotides of E4 mRNA. The deleted sequences were replaced with a fragment derived by PCR which contains open reading frame 6 of Ad2 (nucleotides 34082-33178) and a synthetic poly A addition signal. The topology of the 3' end of the molecule is shown in Figure 34. The sequence of this segment of the molecule will be confirmed. The remainder of the Ad2 viral DNA sequence is



published in Roberts, R.J. in Adenovirus DNA. (W. Oberfler, Martinus Nihoff Publishing, Boston, 1986). The overall size of the Ad2-ORF6/PGK-CFTR vector is 36,336 bp which is 101.3% of full length Ad2. See Table III for the sequence of Ad2-ORF6/PGK-CFTR.

The CFTR transcript is predicted to initiate at one of three closely spaced transcriptional start sites in the cloned PGK promoter (Singer-Sam et al. (1984) *Gene* 32:409-417) at nucleotides 828, 829 and 837 of the recombinant vector (Singer-Sam et al. (1984) *Gene* 32:409-417). A hybrid 5' untranslated region is comprised of 72, 80 or 81 nucleotides of PGK promoter region, 86 nucleotide of linker sequence, and 10 nucleotides derived from the CFTR insert. Transcriptional termination is expected to be directed by the BGH poly A addition signal at recombinant vector nucleotide 5530 yielding an approximately 4.7 kb transcript. The CFTR coding region comprises nucleotides 1010-5454 of the recombinant virus and nucleotides 182, 181 or 173 to 4624, 4623, or 4615 of the PGK-CFTR-BGH mRNA respectively, depending on which transcriptional initiation site is used. Within the CFTR cDNA there are two differences from the published (Riordan et al, *cited supra*) cDNA sequence. An A to C change at position 1990 of the CFTR cDNA (published CFTR cDNA coordinates) which was an error in the original published sequence, and a T to C change introduced at position 936. The change at position 936 is translationally silent but increases the stability of the cDNA when propagated in bacterial plasmids (Gregory et al. (1990) *Nature* 347:382-386; and Cheng et al. (1990) *Cell* 63:827-834). The 3' untranslated region of the predicted CFTR transcript comprises 21 nucleotides of linker sequence and approximately 10 nucleotides of synthetic BGH poly A additional signal.

Although the activity of CFTR can be measured by electrophysiological methods, it is relatively difficult to detect biochemically or immunocytochemically, particularly at low levels of expression (Gregory et al., *cited supra*; and Denning et al. (1992) *J. Cell Biol.* 118:551-559). A high expression level reporter gene encoding the *E. coli* β galactosidase protein fused to a nuclear localization signal derived from the SV40 T-antigen was therefore constructed. Reporter gene transcription is driven by the powerful CMV early gene constitutive promoter. Specifically, the E1 region of wild type Ad2 between nucleotides 357-3498 has been deleted and replaced it with a 515 bp fragment containing the CMV promoter and a 3252 bp fragment encoding the β galactosidase gene.

Regulatory Characteristics of the Elements of the AD2-ORF6/PGK-CFTR

In general terms, the vector is similar to several earlier adenovirus vectors encoding CFTR but it differs in three specific ways from the Ad2/CFTR-1 construct.

PGK Promoter

Transcription of CFTR is from the PGK promoter. This is a promoter of only moderate strength but because it is a so-called house keeping promoter we considered it more likely to be capable of long term albeit perhaps low level expression. It may also be less

likely to be subject to "shut-down" than some of the very strong promoters used in other studies especially with retroviruses. Since CFTR is not an abundant protein longevity of expression is probably more critical than high level expression. Expression from the PGK promoter in a retrovirus vector has been shown to be long lasting (Apperley et al. (1991)

5 *Blood* 78:310-317).

Polyadenylation Signal

Ad2-ORG6/PGK-CFTR contains an exogenous poly A addition signal after the CFTR coding region and prior to the protein IX coding sequence of the Ad2 E1 region. Since protein is believed to be involved in packaging of virions, this coding region was retained. Furthermore, since protein IX is synthesized from a separate transcript with its own promoter, to prevent possible promoter occlusion at the protein IX promoter, the BGH poly A addition signal was inserted. There is indirect evidence that promoter occlusion can be problematic in that Ad2/CMV β Gal grows to lower viral titers on 293 cells than does Ad2/ β gal-1. These constructs are identical except for the promoter used for β galactosidase expression. Since the CMV promoter is much stronger than the E1a promoter it is probable that abundant transcription from the CMV promoter through the β galactosidase DNA into the protein IX coding region reduces expression of protein IX from its own promoter by promoter occlusion and that this is responsible for the lower titer of Ad2/CMV- β gal obtained.

Alterations of the E4 Region

A large portion of the E4 region of the Ad2 genome has been deleted for two reasons. The first reason is to decrease the size of the vector used or expression of CFTR. Adenovirus vectors with genomes much larger than wild type are packaged less efficiently and are therefore difficult to grow to high titer. The combination of the deletions in the E1 and E4 regions in Ad2-ORG6/PGK-CFTR reduce the genome size to 101% of wild type. In practice it is straightforward to prepare high titer lots of this virus.

The second reason to remove E4 sequences relates to the safety of adenovirus vectors. A goal of these studies is to remove as many viral genes as possible to inactivate the Ad2 virus backbone in as many ways as possible. The OF 6/7 gene of the E4 region encodes a protein that is involved in activation of the cellular transcription factor E2-F which is in turn implicated in the activation of the E2 region of adenovirus (Hemstrom et al. (1991) *J. Virol.* 65:1440-1449). Therefore removal of ORF6/7 from adenovirus vectors may provide a further margin of safety at least when grown in non-proliferating cells. The removal of the E1 region already renders such vectors disabled, in part because E1a, if present, is able to displace E2-F from the retinoblastoma gene product, thereby also contributing to the stimulation of E2 transcription. The ORF6 reading frame of Ad2 was added back to the E1-E4 backbone of the Ad2-ORG6/PGK-CFTR vector because ORF6 function is essential for production of the recombinant virus in 293 cells. ORF6 is believed to be involved in DNA replication, host

2145641

- 59 -

cell shut off and late mRNA accumulation in the normal adenovirus life cycle. The E1-E4-ORF6⁺ backbone Ad2 vector does replicate in 293 cells.

The promoter/enhancer use to drive transcription of ORF6 of E4 is the endogenous E4 promoter. This promoter requires E1a for activation and contains E1a core enhancer elements and SP1 transcription factor binding sites (reviewed in Berk, A.J. (1986) *Ann. Rev. Genet.* 20:75-79).

Replication Origin

The only replication origins present in Ad2-ORF6/PGK-CFTR are those present in the Ad2 parent genome. Replication of Ad2-ORF6/PGK-CFTR sequences has not been detected except when complemented with wild type E1 activity.

Steps Used to Derive the DNA Construct

Construction of the recombinant Ad2-ORF6/PGK-CFTR virus was accomplished by *in vivo* recombination of Ad2-ORF6 DNA and a plasmid containing the 5' 10.7 kb of adenovirus engineered to have an expression cassette encoding the human CFTR cDNA driven by the PGK promoter and a BGH poly A signal in place of the E1 coding region.

The generation of the plasmid, pBRAd2/PGK-CFTR is described here. The starting plasmid contains an approximately 7.5 kb insert cloned into the ClaI and BamHI sites of pBR322 and comprises the first 10,680 nucleotides of Ad2 with a deletion of the Ad2 sequences between nucleotides 356 and 3328. This plasmid contains a CMV promoter inserted into the ClaI and SpeI sites at the region of the E1 deletion and is designated pBRAd2/CMV. The plasmid also contains the Ad2 5' ITR, packaging and replication sequences and E1 enhancer. The E1 promoter, E1a and most of E1b coding region has been deleted. The 3' terminal portion of the E1b coding region coincides with the pIX promoter which was retained. The CMV promoter was removed and replaced with the PGK promoter as a ClaI and SpeI fragment from the plasmid PGK-GCR. The resulting plasmid, pBRAd2/PGK, was digested with AvrII and BstBI and the excised fragment replaced with the SpeI to BstBI fragment from the plasmid construct pAd2E1a/CFTR. This transferred a fragment containing the CFTR cDNA, BGH poly A signal and the Ad2 genomic sequences from 3327 to 10,670. The resulting plasmid is designated pBRAd2/PGK-CFTR. The CFTR cDNA fragment was originally derived from the plasmid pCMV-CFTR-936C using restriction enzymes SpeI and Ecl136II. pCMV-CFTR-936C consists of a minimal CFTR cDNA encompassing nucleotides 123-4622 of the published CFTR sequence cloned into the multiple cloning site of pRC/CMV (Invitrogen Corp.) using synthetic linkers. The CFTR cDNA within this plasmid has been completely sequenced.

The Ad2 backbone virus with the E4 region that expresses only open reading frame 6 was constructed as follows. A DNA fragment encoding ORF6 (Ad2 nucleotides 34082-33178) was derived by PCR with ORF6 specific DNA primers. Additional sequences

supplied by the oligonucleotides include cloning sites at the 5' and 3' ends of the PCR fragment. (ClaI and BamHI respectively) and a poly A addition sequence AATAAA at the 3' end to ensure correct polyadenylation of ORF6 mRNA. The PCR fragment was cloned into pBluescript (Stratagene) along with an Ad2 fragment (nucleotides 35937-35577) containing the inverted terminal repeat, E4 promoter, E4 mRNA cap sites and first 32-37 nucleotides of E4 mRNA to create pORF6. A SalI-BamHI fragment encompassing the ITR and ORF6 was used to replace the SalI-BamHI fragment encompassing the ITR and E4 deletion in pAdΔE4 contains the 3' end of Ad2 from a SpeI site to the 3' ITR (nucleotides 27123-35937) and is deleted for all E4 sequences including the promoter and poly A signal (nucleotides 32815-35641). The resulting construct, pAdE4ORF6 was cut with PacI and ligated to Ad2 DNA digested with PacI nucleotide 28612). 293 cells were transfected with the ligation reaction to generate virus containing only open reading frame 6 from the E4 region.

In Vitro Studies with Ad2-ORF6/PGK-CFTR

The ability of Ad2-ORF6/PGK-CFTR to express CFTR in several cell lines, including human HeLa cells, human 293 cells, and primary cultures of normal and CF human airway epithelia was tested. As an example, the results from the human 293 cells is related here. When human 293 cells were grown on culture dishes, the vector was able to transfer CFTR cDNA and express CFTR as assessed by immunoprecipitation and by functional assays of halide efflux. Gregory, R.J. et al. (1990) *Nature* 347:382-386; Cheng, S.H. et al. (1990) *Cell* 63:827-834. More specifically, procedures for preparing cell lysates, immunoprecipitation of proteins using anti-CFTR antibodies, one-dimensional peptide analysis and SDS-polyacrylamide gel electrophoresis were as described by Cheng et al. Cheng, S.H. et al. (1990) *Cell* 63:827-834. Halide efflux assays were performed as described by Cheng, S.H. et al. (1991) *Cell* 66:1027-1036. cAMP-stimulated CFTR chloride channel activity was measured using the halide sensitive fluorophore SPQ in 293 cells treated with 500 IU/cell Ad2-ORF6/PGK-CFTR. Stimulation of the infected cells with forskolin (20 μM) and IBMX (100 μM) increased SPQ fluorescence indicating the presence of functional chloride channels produced by the vector.

Additional studies using primary cultures of human airway (nasal polyp) epithelial cells (from CF patients) infected with Ad2-ORF6/PGK-CFTR demonstrated that Ad2-ORF6/PGK-CFTR infection of the nasal polyp epithelial cells resulted in the expression of cAMP dependent Cl⁻ channels. Figure 35 is an example of the results obtained from such studies. Primary cultures of CF nasal polyp epithelial cells were infected with Ad2-ORF6/PGK-CFTR at multiplicities of 0.3, 3, and 50. Three days post infection, monolayers were mounted in Ussing chambers and short-circuit current was measured. At the indicated times: (1) 10 μM amiloride, (2) cAMP agonists (10 μM forskolin and 100 μM IBMX), and (3) 1 mM diphenylamine-2-carboxylate were added to the mucosal solution.

2145641

- 61 -

In Vivo Studies with Ad2-ORF6/PGK-CFTRVirus preparation

Two preparations of Ad2-ORF6/PGK-CFTR virus were used in this study. Both were prepared at Genzyme Corporation, in a Research Laboratory. The preparations were purified on a CsCl gradient and then dialyzed against tris-buffered saline to remove the CsCl. The preparation for the first administration (lot #2) had a titer of 2×10^{10} IU/ml. The preparation for the second administration (lot #6) had a titer of 4×10^{10} IU/ml.

10 Animals

Three female Rhesus monkeys, *Macaca mulatta*, were used for this study. Monkey C (#20046) weighed 6.4 kg. Monkey D (#20047) weighed 6.25 kg. Monkey E (#20048) weighed 10 kg. The monkeys were housed in the University of Iowa at least 360 days before the start of the study. The animals were maintained with free access to food and water throughout the study. The animals were part of a safety study and efficacy study for a different viral vector (Ad2/CFTR-1) and they were exposed to 3 nasal viral instillation throughout the year. The previous instillation of Ad2/CFTR-1 was performed 116 days prior to the initiation of this study. All three Rhesus monkeys had an anti-adenoviral antibody response as detected by ELISA after each viral instillation. There are no known contaminants that are expected to interfere with the outcome of this study. Fluorescent lighting was controlled to automatically provide alternate light/dark cycles of approximately 12 hours each. The monkeys were housed in an isolation room in separate cages. Strict respiratory and body fluid isolation precautions were taken.

25 Virus administration

For application of the virus, the monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg). The entire epithelium of one nasal cavity in each monkey was used for this study. A foley catheter (size 10) was inserted through each nasal cavity into the pharynx, the balloon was inflated with a 2-3 ml of air, and then pulled anteriorly to obtain a tight occlusion at the posterior choana. The Ad2-ORF6/PGK-CFTR virus was then instilled slowly into the right nostril with the posterior balloon inflated. The viral solution remained in contact with the nasal mucosa for 30 min. The balloons were deflated, the catheters were removed, and the monkeys were allowed to recover from anesthesia.

On the first administration, the viral preparation had a titer of 2×10^{10} IU/ml and each monkey received approximately 0.3 ml. Thus the total dose applied to each monkey was approximately 6.5×10^9 IU. This total dose is approximately half the highest dose proposed for the human study. When considered on a IU/kg basis, a 6 kg monkey received a dose approximately 3 times greater than the highest proposed dose for a 60 kg human.

Timing of evaluations.

The animals were evaluated on the day of administration, and on days 3, 7, 24, 38, and 44 days after infection. The second administration of virus occurred on day 44. The monkeys were evaluated on day 48 and then on days 55, 62, and 129.

- 5 For evaluations, monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg). To obtain nasal epithelial cells after the first viral administration, the nasal mucosa was first impregnated with 5 drops of Afrin (0.05% oxymetazoline hydrochloride, Schering-Plough) and 1 ml of 2% Lidocaine for 5 minutes. A cytobrush was then used to gently rub the mucosa for about 3 sec. To obtain pharyngeal epithelial swabs, a cotton-tipped
10 applicator was rubbed over the back of the pharynx 2-3 times. The resulting cells were dislodged from brushes or applicators into 2 ml of sterile PBS. After the second administration of Ad2-ORF6/PGK-CFTR, the monkeys were followed clinically for 3 weeks, and mucosal biopsies were obtained from the monkeys medial turbinate at days 4, 11 and 18.

15 Animal evaluation.

- Animals were evaluated daily for evidence of abnormal behavior of physical signs. A record of food and fluid intake was used to assess appetite and general health. Stool consistency was also recorded to check for the possibility of diarrhea. At each of the evaluation time points, rectal temperature, respiratory rate, and heart rate were measured.
20 The nasal mucosa, conjunctivas and pharynx were visually inspected. The monkeys were also examined for lymphadenopathy.

Hematology and serum chemistry

- Venous blood from the monkeys was collected by standard venipuncture technique.
25 Blood/serum analysis was performed in the clinical laboratory of the University of Iowa Hospitals and Clinics using a Hitachi 737 automated chemistry analyzer and a Technicom H6 automated hematology analyzer.

Serology

- 30 Sera from the monkeys were obtained and anti-adenoviral antibody titers were measured by ELISA. For the ELISA, 50 ng/well of killed adenovirus (Lee Biomolecular Research Laboratories, San Diego, Ca) was coated in 0.1M NaHCO₃ at 4° C overnight on 96 well plates. The test samples at appropriate dilutions were added, starting at a dilution of 1/50. The samples were incubated for 1 hour, the plates washed, and a goat anti-human IgG
35 HRP conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 hour. The plates were washed and O-Phenylenediamine (OPD) (Sigma Chemical Co., St. Louis, MO) was added for 30 min. at room temperature. The assay was stopped with 4.5 M H₂SO₄ and read at 490 nm on a Molecular Devices microplate reader. The titer was calculated as the product of the reciprocal of the initial dilution and the reciprocal of the



2145641

dilution in the last well with an OD>0.100. Nasal washings from the monkeys were obtained and anti-adenoviral antibody titers were measured by ELISA, starting at a dilution of 1/4.

Nasal Washings.

5 Nasal washings were obtained to test for the possibility of secretory antibodies that could act as neutralizing antibodies. Three ml of sterile PBS was slowly instilled into the nasal cavity of the monkeys, the fluid was collected by gravity. The washings were centrifuged at 1000 RPM for 5 minutes and the supernatant was used for anti-adenoviral, and neutralizing antibody measurement.

10

Cytology

Cells were obtained from the monkey's nasal epithelium by gently rubbing the nasal mucosa for about 3 seconds with a cytobrush. The resulting cells were dislodged from the brushes into 2 ml of PBS. The cell suspension was spun at 5000 rpm for 5 min. and
15 resuspended in 293 media at a concentration of 10^6 cells/ml. Forty μ l of the cell suspension was placed on slides using a Cytospin. Cytospin slides were stained with Wright's stain and analyzed for cell differential using light microscopy.

Culture for Ad2-ORF6/PEK-CFTR

20 To assess for the presence of infectious viral particles, the supernatant from the nasal brushings and pharyngeal swabs of the monkeys were used. Twenty-five μ l of the supernatant was added in duplicate to 293 cells. 293 cells were used at 50% confluence and were seeded in 96 well plates. 293 cells were incubated for 72 hours at 37°C, then fixed with a mixture of equal parts of methanol and acetone for 10 min and incubated with an FITC
25 label anti-adenovirus monoclonal antibodies (Chemicon, Light Diagnostics, Temecuca, Ca) for 30 min. Positive nuclear immunofluorescence was interpreted as positive culture.

Immunocytochemistry for the detection of CFTR.

Cells were obtained by brushing. Eighty μ l of cell suspension were spun onto gelatin-coated slides. The slides were allowed to air dry, and then fixed with 4% paraformaldehyde. The cells were permeabilized with 0.2 Triton-X (Pierce, Rockford, Il) and then blocked for 60 minutes with 5% goat serum (Sigma, Mo). A pool of monoclonal antibodies (M13-1, M1-4, and M6-4) (Gregory et al., (1990) *Nature* 347:382-386); Denning et al., (1992) *J. Cell Biol.* 118:(3) 551-559); Denning et al., (1992) *Nature* 358:761-764) were added and incubated for
35 12 hours. The primary antibody was washed off and an antimouse biotinylated antibody (Biomed, Foster City, Ca) was added. After washing, the secondary antibody, streptavidin FITC (Biomed, Foster City, Ca) was added and the slides were observed with a laser scanning confocal microscope.

2145641

Biopsies

To assess for histologic evidence of safety, nasal medial turbinate biopsies were obtained on day 4, 11 and 18 after the second viral administration as described before (Zabner et al (1993) Human Gene Therapy, in press). Nasal biopsies were fixed in 4% formaldehyde and H&E stained sections were reviewed.

RESULTS

Studies of efficacy.

To directly assess the presence of CFTR, cells obtained by brushing were plated onto slides by cytopspin and stained with antibodies to CFTR. A positive reaction is clearly evident in cells exposed to Ad2-ORF6/PGK-CFTR. The cells were scored as positive by immunocytochemistry when evaluated by a reader blinded to the identity of the samples. Cells obtained prior to infection and from other untreated monkeys were used as negative controls. Figures 36A-36D, 37A-37D, and 38A-38D show examples from each monkey.

Studies of safety

None of the monkeys developed any clinical signs of viral infections or inflammation. There were no visible abnormalities at days 3, 4, 7 or on weekly inspection thereafter. Physical examination revealed no fever, lymphadenopathy, conjunctivitis, coryza, tachypnea, or tachycardia at any of the time points. There was no cough, sneezing or diarrhea. The monkeys had no fever. Appetites and weights were not affected by virus administration in either monkey. The data are summarized in Figures 39A-39C.

The presence of live virus was tested in the supernatant of cell suspensions from swabs and brushes from each nostril and the pharynx. Each supernatant was used to infect the virus-sensitive 293 cell line. Live virus was never detected at any of the time points. The rapid loss of live virus suggests that there was no viral replication.

The results of complete blood counts, sedimentation rate, and clinical chemistries are shown in Figure 40A-40C. There was no evidence of a systemic inflammatory response or other abnormalities of the clinical chemistries.

Epithelial inflammation was assessed by cytological examination of Wright-stained cells (cytopspin) obtained from brushings of the nasal epithelium. The percentage of neutrophils and lymphocytes from the infected nostrils were compared to those of the control nostrils and values from four control monkeys. Wright stains of cells from nasal brushing were performed on each of the evaluation days. Neutrophils and lymphocytes accounted for less than 5% of total cells at all time points. The data are shown in Figure 41. The data indicate that administration of Ad2-ORF6/PGK-CFTR caused no change in the distribution or number of inflammatory cells at any of the time points following virus administration,

2145641

- 65 -

even during a second administration of the virus. The biopsy slides obtained after the second Ad2-ORF6/PGK-CFTR administration were reviewed by an independent pathologist, who found no evidence of inflammation or any other cytopathic effects. Figures 42 to 44 show an example from each monkey.

5 Figures 45A-45C shows that all three monkeys had developed antibody titers to adenovirus prior to the first infection with Ad2-ORF6/PGK-CFTR (Zabner et al. (1993) *Human Gene Therapy* (in press)). Antibody titers measured by ELISA rose within one week after the first and second administration and peaked at day 24. No anti-adenoviral antibodies were detected by ELISA or neutralizing assay in nasal washings of any of the monkeys.

10 These results combined with demonstrate the ability of a recombinant adenovirus encoding CFTR (Ad2-ORF6/PGK-CFTR) to express CFTR cDNA in the airway epithelium of monkeys. These monkeys have been followed clinically for 12 months after the first viral administration and no complications have been observed.

15 The results of the safety studies are encouraging. No evidence of viral replication was found; infectious viral particles were rapidly cleared. The other major consideration for safety of an adenovirus vector in the treatment of CF is the possibility of an inflammatory response. The data indicate that the virus generated an antibody response, but despite this, no evidence of a systemic or local inflammatory response was observed. The cells obtained by brushings and swabs were not altered by virus application. Since these Monkeys had been
20 previously exposed three times to Ad2/CFTR-1, these data suggest that at least five sequential exposures of airway epithelium to adenovirus does not cause a detrimental inflammatory response.

25 These data indicate that Ad2-ORF6/PGK-CFTR can effectively transfer CFTR cDNA to airway epithelium and direct the expression of CFTR. They also indicate that transfer and expression is safe in primates.

Equivalents

30 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

TABLE I

| <u>Mutant</u> | <u>CE</u> | <u>Exon</u> | <u>CFTR Domain</u> | <u>A</u> | <u>B</u> |
|---------------|-----------|-------------|--------------------|----------|----------|
| Wild Type | | | | - | + |
| R334W | Y | 7 | TM6 | - | + |
| K464M | N | 9 | NBD1 | - | + |
| Δ1507 | Y | 10 | NBD1 | - | + |
| ΔF508 | Y | 10 | NBD1 | - | + |
| F508R | N | 10 | NBD1 | - | + |
| S549I | Y | 11 | NBD1 | - | + |
| G551D | Y | 11 | NBD1 | - | + |
| N894,900Q | N | 15 | ECD4 | + | - |
| K1250M | N | 20 | NBD2 | - | + |
| Tth111 | N | 22 | NB-Term | - | + |

Table II.

2145641

| 10 | 20 | 30 | 40 | 50 | 60 |
|--------------------------------------------------------------------|-----|-----|-----|-----|--------------|
| CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT | | | | | |
| GTAGTAGTTA TTATATGGAA TAAACCTAA CTTCGGTTAT ACTATTACTC CCCCACCTCA | | | | | |
| ____INVERTED TERMINAL REPETITION-ORIGIN OF REPLICATION____ | | | | | 60> |
| 70 | 80 | 90 | 100 | 110 | 120 |
| TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT | | | | | |
| AACACTGCAC CGCGCCCCGC ACCCTTGCCC CGCCCACTGC ATCATCACAC CGCCTTCACA | | | | | |
| ____INVERTED TERMINAL REPETITION-ORIGIN OF R____> | | | | | |
| 130 | 140 | 150 | 160 | 170 | 180 |
| GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGCCGGATG TGGTAAAAGT GACGTTTTTG | | | | | |
| CTACAACGTT CACACCGCCT TGTGTACATT CGCGGCCTAC ACCATTTTCA CTGCAAAAAC | | | | | |
| 190 | 200 | 210 | 220 | 230 | 240 |
| GTGTGCGCCG GTGTATACGG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG | | | | | |
| CACACGCGGC CACATATGCC CTTCAGTGT AAAAGCGCGC CAAAATCCGC CTACAACATC | | | | | |
| ____b____E1A ENHANCER AND VIRAL PACKAGING DOMAIN____ | | | | | 50> |
| 250 | 260 | 270 | 280 | 290 | 300 |
| TAAATTTGGG CGTAACCAAG TAATGTTTGG CCATTTTCGC GGGAAACTG AATAAGAGGA | | | | | |
| ATTTAAACCC GCATTGGTTC ATTACAACC GGTAAAGCG CCCTTTTGAC TTATTCTCCT | | | | | |
| ____60_b_E1A ENHANCER AND VIRAL PACKAGING DOMAIN_0_b____ | | | | | 110> |
| 310 | 320 | 330 | 340 | 350 | 360 |
| AGTGAAATCT GAATAATTCT GTGTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG | | | | | |
| TCACCTTAGA CTTATTAAGA CACAATGAGT ATCGCGCATT ATAAACAGAT CCGGCGCGCC | | | | | |
| ____120_b_E1A ENHANCER AND VIRAL PACKAGING DOMAIN_0_b____ | | | | | 170> |
| 370 | 380 | 390 | 400 | 410 | 420 |
| GACTTTGACC GTTTACGTGS AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC | | | | | |
| CTGAAGCTGG CAAATGCACC TCTGAGCGGG TCCACAAAA GAGTCCACAA AAGGCGCAAG | | | | | |
| ____E1A ENHANCER A_90> | | | | | |
| ____c____10_E1A PROMOTER REGION_0_c____ | | | | | 40> |
| 430 | 440 | 450 | 460 | 470 | 480 |
| CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGCTGACG CGCAGTGTAT TTATACCCGG | | | | | |
| GCCGAGTTTC AACCACAAAA TAATAATATC ACTCGACTGC GCGTCACATA AATATGGCCC | | | | | |
| ____50_c____60_E1A PROMOTER REGION_c____ | | | | | 90_c____100> |
| 490 | 500 | 510 | 520 | 530 | 540 |
| TQAGTTCCCTC AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC | | | | | |
| ACTCAAGGAG TTCTCCGGTG AGAACTCAG GTCGCTCATC TCAAAAGAGG AGGCTCGGCC | | | | | |
| ____h____HYBRED E1A-CFTR-E1B MESSAGE____> | | | | | |
| ____E1A PROMOTER_120> | | | | | |
| ____6____E1A mRNA 5' UNTRANSLATED_6____ | | | | | 40> |
| 550 | 560 | 570 | 580 | 590 | 600 |
| TCCGAGCTAG TAACGCCCGC CAGTGCTGTG CAGATATCAA AGTCGACGGT ACCCGAGAGA | | | | | |
| AGGCTCGATC ATTGCCGGCG GTCACACGAC GTCTATAGTT TCAGGTGCA TGGGCTCTCT | | | | | |

_____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____>
 _____e_____10_____SYNTHETIC LINKER SEQUENCES_____40_____e_____>
 _____130>

610 620 630 640 650 660
 CCATGCAGAG GTCGCCTCTG GAAAAGGCCA GCGTTGTCTC CAAACTTTTT TTCAGCTGGA
 GGTACGTCTC CAGCGGAGAC CTTTTCCGGT CGCAACAGAG GTTTGAAAAA AAGTCGACCT
 M Q R S P L E K A S V V S K L F F S W>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____>
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____140i_____123 TO 4622 OF HUMAN CFTR CDNA_____180i_____190>

670 680 690 700 710 720
 CCAGACCAAT TTTGAGGAAA GGATACAGAC AGCGCCTGGA ATTGTCAGAC ATATACCAAA
 GGTCTGGTTA AAACCTCTTT CCTATGTCTG TCGCGGACCT TAACAGTCTG TATATGGTTT
 T R P I L R K G Y R Q R L E L S D I Y Q>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____>
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____200i_____123 TO 4622 OF HUMAN CFTR CDNA_____240i_____250>

730 740 750 760 770 780
 TCCCTTCTGT TGATTCTGCT GACAATCTAT CTGAAAAATT GGAAAGAGAA TGGGATAGAG
 AGGGAAGACA ACTAAGACGA CTGTTAGATA GACTTTTAA CCTTTCTCTT ACCCTATCTC
 I P S V D S A D N L S E K L E R E W D R>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____>
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____260i_____123 TO 4622 OF HUMAN CFTR CDNA_____300i_____310>

790 800 810 820 830 840
 AGCTGGCTTC AAAGAAAAAT CCTAACTCA TTAATGCCCT TCGGCGATGT TTTTCTGGA
 TCGACCGAAG TTTCTTTTAA GGATTTGAGT AATTACGGGA AGCCGCTACA AAAAAGACCT
 E L A S K K N P K L I N A L R R C F F W>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____>
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____320i_____123 TO 4622 OF HUMAN CFTR CDNA_____360i_____370>

850 860 870 880 890 900
 GATTTATGTT CTATGGAATC TTTTATATT TAGGGGAAGT CACCAAAGCA GTACAGCCTC
 CTAANTACAA GATACCTTAG AAAAATATAA ATCCCCITCA GTGGTTTCGT CATGTCGGAG
 R F M F Y G I F L Y L G E V T K A V Q P>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____>
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____380i_____123 TO 4622 OF HUMAN CFTR CDNA_____420i_____430>

910 920 930 940 950 960
 TCTTACTGGG AAGAATCATA GCTTCCTATG ACCCGGATAA CAAGGAGGAA CGCTCTATCG
 AGAATGACCC TTCTTAGTAT CGAAGGATAC TCGGCTATT GTTCCTCCTT GCGAGATAGC
 L L L G R I I A S Y D P D N K E E R S I>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____>
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____440i_____123 TO 4622 OF HUMAN CFTR CDNA_____480i_____490>

970 980 990 1000 1010 1020
 CGATTTATCT AGGCATAGGC TTATGCTTTC TCTTTATTGT GAGGACACTG CTCCTACACC

GCTAAATAGA TCCGTATCCG AATACGGAAG AGAATAACA CTCCTGTGAC GAGGATGTGG
 A I Y L G I G L C L L F I V R T L L L H>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____500i____123 TO 4622 OF HUMAN CFTR CDNA____540i____550>

1030 1040 1050 1060 1070 1080

CAGCCATTTT TGGCCTTCAT CACATTGGAA TGCAGATGAG AATAGCTATG TTTAGTTTGA
 GTCGGTAAAA ACCGGAAGTA GTGTAACCTT ACGTCTACTC TTATCGATAC AAATCAAACCT
 P A I F G L H H I G M Q M R I A M F S L>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____560i____123 TO 4622 OF HUMAN CFTR CDNA____600i____610>

1090 1100 1110 1120 1130 1140

TTTATAAGAA GACTTTAAAG CTGTCAAGCC GTGTTCTAGA TAAAATAAGT ATTGGACAAC
 AAATATTCTT CTGAAATTC GACAGTTCGG CACAAGATCT ATTTTATTCA TAACCTGTTG
 I Y K K T L K L S S R V L D K I S I G Q>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____620i____123 TO 4622 OF HUMAN CFTR CDNA____660i____670>

1150 1160 1170 1180 1190 1200

TTGTTAGTCT CCTTTCCAAC AACCTGAACA AATTTGATGA AGGACTTGCA TTGGCACATT
 AACATCAGA GGAAGGTTG TTGGACTTGT TAAACTACT TCCTGAACGT AACCGTGTA
 L V S L L S N N L N K F D E G L A L A H>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____680i____123 TO 4622 OF HUMAN CFTR CDNA____720i____730>

1210 1220 1230 1240 1250 1260

TCGTGTGGAT CGCTCCTTTG CAAGTGGCAC TCCTCATGGG GCTAATCTGG GAGTTGTTAC
 AGCACACCTA GCGAGGAAC GTTCACCGTG AGGAGTACCC CGATTAGACC CTCAACAATG
 F V W I A P L Q V A L L M G L I W E L L>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____740i____123 TO 4622 OF HUMAN CFTR CDNA____780i____790>

1270 1280 1290 1300 1310 1320

AGGCGTCTGC CTTCTGTGGA CTTCGTTTCC TGATAGTCTT TGCCCTTTTT CAGGCTGGGC
 TCCGCAGACG GAAGACACCT GAACCAAAGG ACTATCAGGA ACGGGAAGAA GTCCGACCCG
 Q A S A F C G L G F L I V L A L F Q A G>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____800i____123 TO 4622 OF HUMAN CFTR CDNA____840i____850>

1330 1340 1350 1360 1370 1380

TAGGGAGAAT GATGATGAAG TACAGAGATC AGAGAGCTGG GAAGATCAGT GAAAGACTTG
 ATCCCTCTTA CTACTACTTC ATGTCTCTAG TCTCTCGACC CTTCTAGTCA CTTTCTGAAC
 L G R M M M K Y R D Q R A G K I S E R L>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____860i____123 TO 4622 OF HUMAN CFTR CDNA____900i____910>

1390 1400 1410 1420 1430 1440

TGATTACCTC AGAAATGATT GAAAACATCC AATCTGTTAA GGCATACTGC TCGGAAGAAG
 ACTAATGGAG TCTTTACTAA CTTTGTAGG TTAGACAATT CCGTATGACG ACCCTTCTTC
 V I T S E M I E N I Q S V K A Y C W E E
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 920i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 960i _____ 970>

1450 1460 1470 1480 1490 1500
 CAATGGAAAA AATGATTGAA AACTTAAGAC AAACAGAACT GAAACTGACT CGGAAGGCAG
 GTTACCTTTT TTACTAAGTT TTGAATCTG TTTGTCTTGA CTTTGACTGA GCCTTCCGTC
 A M E K M I E N L R Q T E L K L T R K A
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 980i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 1020i _____ 1030>

1510 1520 1530 1540 1550 1560
 CCTATGTGAG ATACTTCAAT AGCTCAGCCT TCTTCTTCTC AGGGTTCTTT GTGGTGTTTT
 GGATACACTC TATGAAGTTA TCGAGTCGGA AGAAGAAGAG TCCCAAGAAA CACCACAAAA
 A Y V R Y F N S S A F F F S G F F V V F
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 1040i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 1080i _____ 1090>

1570 1580 1590 1600 1610 1620
 TATCTGTGCT TCCCTATGCA CTAATCAAAG GAATCATCCT CCGGAAAATA TTCACCA
 ATAGACAGGA AGGGATACGT GATTAGTTTC CTTAGTAGGA GGCCTTTTAT AAGTGGTGGT
 L S V L P Y A L I R G I I L R K I F T T
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 1100i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 1140i _____ 1150>

1630 1640 1650 1660 1670 1680
 TCTCATTCTG CATTGTTCTG CGCATGGCGG TCACTCGGCA ATTTCCCTGG GCTGTACAAA
 AGAGTAAGAC GTAACAAGAC GCGTACCGCC AGTGAGCCGT TAAAGGGACC CGACATGTTT
 I S F C I V L R M A V T R Q F P W A V Q
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 1160i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 1200i _____ 1210>

1690 1700 1710 1720 1730 1740
 CATGCTATCA CTCTCTTGGC GCAATAAACA AAATACAGGA TTTETTACAA AAGCAAGAAT
 GTACCATACT GAGAGAACCT EGTATTGTG TTTATGTCCT AAAGAATGTT TTCGTTCTTA
 T W Y D S L G A I N K I Q D F L Q K Q E
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 1220i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 1260i _____ 1270>

1750 1760 1770 1780 1790 1800
 ATAAGACATT GGAATATAAC TTAACCACTA CAGAAGTAGT GATGGAGAAT GTAACAGCCT
 TATTCTGTAA CCTTATATTG AATTGCTGAT GTCTTCATCA CTACCTCTTA CATTGTCCGA
 Y K T L E Y N L T T T E V V M E N V T A
 _____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____>
 _____ h _____ HYBRID ELA-CFTR-ELB MESSAGE _____ h _____>
 _____ 1280i _____ 123 TO 4622 OF HUMAN CFTR CDNA _____ 1320i _____ 1330>

1810 1820 1830 1840 1850 1866

2145641

-71-

TCTGGGAGGA GGGATTGTTGGG GAATTATTTG AGAAAGCAA ACAAAACAAT AACAAATAGAA
 AGACCCTCCT CCCTAAACCC CTTAATAAAC TCITTCGTTT TGTITTTGTTA TTGTTATCTT
 F W E E G F G E L F E K A K Q N N N N R>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1340i____123 TO 4622 OF HUMAN CFTR CDNA____1380i____1390>

1870 1880 1890 1900 1910 1920

AAACTTCTAA TGGTGATGAC AGCCTCTTCT TCAGTAATTT CTCACTTCTT GGTACTCCTG
 TTTGAAGATT ACCACTACTG TCGGAGAAGA AGTCATTAAA GAGTGAAGAA CCATGAGGAC
 K T S N G D D S L F F S N F S L L G T P>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1400i____123 TO 4622 OF HUMAN CFTR CDNA____1440i____1450>

1930 1940 1950 1960 1970 1980

TCCTGAAAGA TATTAATTTT AAGATAGAAA GAGGACAGTT GTTGGCGGTT GCTGGATCCA
 AGGACTTTCT ATAATTAAAG TTCTATCTTT CTCCTGTCAA CAACCGCCAA CGACCTAGGT
 V L K D I N F K I E R G Q L L A V A G S>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1460i____123 TO 4622 OF HUMAN CFTR CDNA____1500i____1510>

1990 2000 2010 2020 2030 2040

CTGGAGCAGG CAAGACTTCA CTTCTAATGA TGATTATGGG AGAACTGGAG CCTTCAGAGG
 GACCTCGTCC GTTCTGAAGT GAAGATTACT ACTAATACCC TCTTGACCTC GGAAGTCTCC
 T G A G K T S L L M M I M G E L E P S E>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1520i____123 TO 4622 OF HUMAN CFTR CDNA____1560i____1570>

2050 2060 2070 2080 2090 2100

GTAAATTTAA GCACAGTGGA AGAATTTTCA TCTGTTCTCA GTTTTCCTGG ATTATGCCTG
 CATTTTAATT CGTGTCACCT TCTTAAAGTA AGACAAGACT CAAAAGGACC TAATACGGAC
 G K I K H S G R I S F C S Q F S W I M P>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1580i____123 TO 4622 OF HUMAN CFTR CDNA____1620i____1630>

2110 2120 2130 2140 2150 2160

GCACCATTAA AGAAAATATC ATCTTTGGTG TTTCCTATEA TGAATATAGA TACAGAAGCC
 CGTGGTAATT TCTTTTATAG TAGAAACCAC AAAGSATACT ACTTATATCT ATGTCTTCGC
 G T I K E N I I F G V S Y D E Y R Y R S>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1640i____123 TO 4622 OF HUMAN CFTR CDNA____1680i____1690>

2170 2180 2190 2200 2210 2220

TCATCAAAGC ATGCCAACTA GAAGAGGACA TCTCCAAGTT TGCAGAGAAA GACAATATAG
 AGTAGTTTCG TACGGTTGAT CTCTCCTGT AGAGGTTCAA ACGTCTCTTT CTGTTATATC
 V I K A C Q L E E D I S K F A E K D N I>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____1700i____123 TO 4622 OF HUMAN CFTR CDNA____1740i____1750>

-72-

2230 2240 2250 2260 2270 2280
TTCTTGGAGA AGGTGGAATC AACTGAGTG GAGGTCAACG AGCAAGAATT TCTTTAGCAA
AAGAACCCTCT TCCACCTTAG TGTGACTCAC CTCCAGTTGC TCGTTCTTAA AGAAATCGTT
V L G E G G I T L S G G Q R A R I S L A>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____1760i____123 TO 4622 OF HUMAN CFTR CDNA____1800i____1810>
2290 2300 2310 2320 2330 2340
GAGCAGTATA CAAAGATGCT GATTGTATT TATTAGACTC TCCTTTTGGGA TACCTAGATG
CTCGTCATAT GTTCTACGA CTAAACATAA ATAATCTGAG AGGAAAACCT ATGGATCTAC
R A V Y K D A D L Y L L D S P F G Y L D>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____1820i____123 TO 4622 OF HUMAN CFTR CDNA____1860i____1870>
2350 2360 2370 2380 2390 2400
TTTTAACAGA AAAAGAAATA TTGAAAGCT GTGTCTGTAA ACTGATGGCT AACAAAATA
AAAATTGTCT TTTTCTTTAT AAACCTTCGA CACAGACATT TGACTACCGA TTGTTTTGAT
V L T E K E I F E S C V C K L M A N K T>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____1880i____123 TO 4622 OF HUMAN CFTR CDNA____1920i____1930>
2410 2420 2430 2440 2450 2460
GGATTTTGGT CACTTCTAAA ATGGAACATT TAAAGAAAGC TGACAAAATA TTAATTTTGC
CCTAAAACCA GTGAAGATTT TACCTTGTA ATTTCTTTTCG ACTGTTTTAT AATTAAAACG
R I L V T S K M E H L K K A D K I L I L>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____1940i____123 TO 4622 OF HUMAN CFTR CDNA____1980i____1990>
2470 2480 2490 2500 2510 2520
ATGAAGGTAG CAGCTATTTT TATGGGACAT TTTCAGAACT CCAAATCTA CAGCCAGACT
TACTTCCATC GTCGATAAAA ATACCCTGTA AAAGTCTTGA GGTTTTAGAT GTCGGTCTGA
H E G S S Y F Y G T F S E L Q N L Q P D>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____2000i____123 TO 4622 OF HUMAN CFTR CDNA____2040i____2050>
2530 2540 2550 2560 2570 2580
TTAGCTCAAA ACTCATGGGA TGTGATTCTT TCGACCAATT TAGTGCAGAA AGAAGAAATT
AATCGAGTTT TGAGTACCCT AACTAAGAA AGCTGGTTAA ATCAGTCTT TCTTCTTTAA
F S S K L M G C D S F D Q F S A E R R N>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____2060i____123 TO 4622 OF HUMAN CFTR CDNA____2100i____2110>
2590 2600 2610 2620 2630 2640
CAATCCTAAC TGAGACCTTA CACCGTTTCT CATTAGAAGG AGATGCTCCT GTCTCCTGGA
GTTAGGATTG ACTCTGGAAT GTGGCAAAGA GTAATCTTCC TCTACGAGGA CAGAGGACCT
S I L T E T L H R F S L E G D A P V S W>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____2120i____123 TO 4622 OF HUMAN CFTR CDNA____2160i____2170>

2145641

PCT/US93/11667

-73-

2650 2660 2670 2680 2690 2700
 CAGAAACAAA AAAACAATCT TTAAACAGA CTGGAGAGTT TGGGAAAAA AGGAAGAATT
 GTCTTTGTTT TTTTGTAGA AAATTGTCT GACCTCTCAA ACCCCTTTT TCCTTCTTAA
 T E T K K Q S F K Q T G E F G E K R K N>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____2180i____123 TO 4622 OF HUMAN CFTR CDNA____2220i____2230>

2710 2720 2730 2740 2750 2760
 CTATTCTCAA TCCAATCAAC TCTATACGAA AATTTTCCAT TGTGCAAAG ACTCCCTTAC
 GATAAGAGTT AGGTTAGTTG AGATATGCTT TTAAAAGGTA ACACGTTTTC TGAGGGAATG
 S I L N P I N S I R K F S I V Q K T P L>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____2240i____123 TO 4622 OF HUMAN CFTR CDNA____2280i____2290>

2770 2780 2790 2800 2810 2820
 AAATGAATGG CATCGAAGAG GATTCTGATG AGCCTTTAGA GAGAAGGCTG TCCTTAGTAC
 TTTACTTACC GTAGCTTCTC CTAAGACTAC TCGGAAATCT CTCTCCGAC AGGAATCATG
 Q M N G I E E D S D E P L E R R L S L V>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____2300i____123 TO 4622 OF HUMAN CFTR CDNA____2340i____2350>

2830 2840 2850 2860 2870 2880
 CAGATTCTGA GCAGGGAGAG GCGATACTGC CTCGCATCAG CGTGATCAGC ACTGGCCCCA
 GTCTAAGACT CGTCCCTCTC CGCTATGACG GACGCTAGTC GCACTAGTCG TGACCGGGGT
 P D S E Q G E A I L P R I S V I S T G P>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____2360i____123 TO 4622 OF HUMAN CFTR CDNA____2400i____2410>

2890 2900 2910 2920 2930 2940
 CGCTTCAGGC ACGAAGGAGG CAGTCTGTCC TGAACCTGAT GACACACTCA GTTAACCAAG
 GCGAAGTCCG TGCTTCTCTC GTCAGACAGG ACTTGGACTA CTGTGTGAGT CAATTGGTTT
 T L Q A R R R Q S V L N L M T H S V N Q>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____2420i____123 TO 4632 OF HUMAN CFTR CDNA____2460i____2470>

2950 2960 2970 2980 2990 3000
 GTCAGAACAT TCACCGAAAG ACAACAGCAT CCACACGAAA AGTGTCACTG GCCCTCAGG
 CAGTCTTGTA ACTGGCTTTC TSTTGTGCTA GSTGTGCTT TCACAGTGAC CGGGGAGTCC
 G Q N I H R K T T A S T R K V S L A P Q>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____2480i____123 TO 4622 OF HUMAN CFTR CDNA____2520i____2530>

3010 3020 3030 3040 3050 3060
 CAAACTTGAC TGAAGTGCAT ATATATTCAA GAAGGTTATC TCAAGAAACT GGCTTCGAAA
 GTTGAAGTGC ACTTGACCTA TATATAAGTT CTTCCAATAG AGTTCTTTGA CCGAAGCTTT
 A N L T E L D I Y S R R L S Q E T G L E>
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>

_____2540i_____123 TO 4622 OF HUMAN CFTR CDNA_____2580i_____2590>
 3070 3080 3090 3100 3110 3120
 TAAGTGAAGA AATTAACGAA GAAGACTTAA AGGAGTGCCT TTTTGATGAT ATGGAGAGCA
 ATTCACCTTCT TTAATTGCTT CTCTCTGAATT TCCTCAGCGA AAAACTACTA TACCTCTCGT
 I S E E I N E E D L K E C L F D D M E S>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE_____h_____
 _____2600i_____123 TO 4622 OF HUMAN CFTR CDNA_____2640i_____2650>
 3130 3140 3150 3160 3170 3180
 TACCAGCAGT GACTACATGG AACACATACC TTCGATATAT TACTGTCCAC AAGAGCTTAA
 ATGGTCGTCA CTGATGTACC TTGTGTATGG AAGCTATATA ATGACAGGTG TTCTCGAATT
 I P A V T T W N T Y L R Y I T V H K S L>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE_____h_____
 _____2660i_____123 TO 4622 OF HUMAN CFTR CDNA_____2700i_____2710>
 3190 3200 3210 3220 3230 3240
 TTTTGTGCT AATTGGTGC TTAGTAATTT TTCTGGCAGA GGTGGCTGCT TCTTTGGTTG
 AAAACACGA TTAACCACG AATCATTAAG AAGACCGTCT CCACCGACGA AGAAACCAAC
 I F V L I W C L V I F L A E V A A S L V>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE_____h_____
 _____2720i_____123 TO 4622 OF HUMAN CFTR CDNA_____2760i_____2770>
 3250 3260 3270 3280 3290 3300
 TGCTGTGGCT CCTTGGAAC ACTCCTCTTC AAGACAAAGG GAATAGTACT CATAGTAGAA
 ACGACACCGA GGAACCTTTG TGAGGAGAAG TTCTGTTTCC CTTATCATGA GTATCATCTT
 V L W L L G N T P L Q D K G N S T H S R>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE_____h_____
 _____2780i_____123 TO 4622 OF HUMAN CFTR CDNA_____2820i_____2830>
 3310 3320 3330 3340 3350 3360
 ATAACAGCTA TGCACTGATT ATCACCAGCA CCAGTTCGTA TTATGTGTTT TACATTTACG
 TATTGTGAT ACGTCACTAA TAGTGGTCGT GGTCAAGCAT AATACACAAA ATGTAATGC
 N N S Y A V I I T S T S S Y Y V F Y I Y>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE_____h_____
 _____2840i_____123 TO 4622 OF HUMAN CFTR CDNA_____2880i_____2890>
 3370 3380 3390 3400 3410 3420
 TGGGAGTAGC CGACACTTTG CTGCTATGG GATTCTTCAG AGGTCTACCA CTGGTGCATA
 ACCCTCATCG GCTGTGAAAC GAACGATACC CTAAGAAGTC TCCAGATGGT GACCACGTAT
 V G V A D T L L A M G F F R G L P L V H>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____
 _____h_____HYBRID ELA-CFTR-ELB MESSAGE_____h_____
 _____2900i_____123 TO 4622 OF HUMAN CFTR CDNA_____2940i_____2950>
 3430 3440 3450 3460 3470 3480
 CTCTAATCAC AGTGTGCAAA ATTTTACACC ACAAATGTT ACATTCTGTT CTTCAAGCAC
 GAGATTAGTG TCACAGCTTT TAAATGTGG TCTTTTACAA TGTAAGACAA GAAGTTCGTG
 T L I T V S K I L M H K M L H S V L Q A>
 _____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON_____

2145641
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____2960i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3000i_____ 3010>

3490 3500 3510 3520 3530 3540

CTATGTCAAC CCTCAACACG TTGAAAGCAG GTGGGATTCT TAATAGATTG TCCAAAGATA
 GATACAGTTG GGAGTTGTGC AACTTTTCGTC CACCCTAAGA ATTATCTAAG AGGTTTCTAT
 P M S T L N T L K A G G I L N R F S K D>
 _____h_____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____h_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____3020i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3060i_____ 3070>

3550 3560 3570 3580 3590 3600

TAGCAATTTT GGATGACCTT CTGCCTCTTA CCATATTTGA CTTCATCCAG TTGTTATTAA
 ATCGTTAAAA CCTACTGGAA GACGGAGAAT GGTATAAAGT GAAGTAGGTC AACAATAATT
 I A I L D D L L P L T I F D F I Q L L L>
 _____h_____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____h_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____3080i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3120i_____ 3130>

3610 3620 3630 3640 3650 3660

TTGTGATTGG AGCTATAGCA GTTGTCCGAG TTTTACAACC CTACATCTTT GTTGCAACAG
 AACACTAACC TCGATATCGT CAACAGCGTC AAAATGTTGG GATGTAGAAA CAACGTTGTC
 I V I G A I A V V A V L Q P Y I F V A T>
 _____h_____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____h_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____3140i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3180i_____ 3190>

3670 3680 3690 3700 3710 3720

TGCCAGTGAT AGTGGCTTTT ATTATGTTGA GAGCATATTT CCTCCAAACC TCACAGCAAC
 ACGGTCACCTA TCACCGAAAA TAATACAACCT CTCGTATAAA GGAGGTTTGG AGTGTGCGTTG
 V P V I V A F I M L R A Y F L Q T S Q Q>
 _____h_____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____h_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____3200i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3240i_____ 3250>

3730 3740 3750 3760 3770 3780

TCAAACAACCT GGAATCTGAA GGCAGGAGTC CAATTTTCAC TCATCTTGTT ACAAGCTTAA
 AGTTTGTGTA CCTTAGACTT CCGTCCTCAG GTTAAAAGTG AGTAGAACAA TGTTCGAATT
 L K Q L E S E G R S P I F T H L V T S L>
 _____h_____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____h_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____3260i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3300i_____ 3310>

3790 3800 3810 3820 3830 3840

AAGCACTATG GACACTTCGT GCCTTCGGAC GGCAGCCTTA CTTTGAAACT CTGTTCCACA
 TTCCTGATAC CTGTGAAGCA CGGAAGCCTG CCGTCGGAAT GAAACTTTGA GACAACCTGT
 K G L W T L R A F G R Q P Y F E T L F H>
 _____h_____ CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON _____h_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____3320i_____ 123 TO 4622 OF HUMAN CFTR CDNA _____3360i_____ 3370>

3850 3860 3870 3880 3890 3900

AAGCTCTGAA TTTACATACT GCCAACTGGT TCTTGTACCT GTCAACACTG CGCTGTTTCC
 TTCGAGACTT AAATGTATGA CCGTTGACCA AGAACATGGA CAGTTGTGAC GCGAECGAAG
 K A L N L H T A N W F L Y L S T L R W F>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3380i___123 TO 4622 OF HUMAN CFTR CDNA___3420i___3430>

3910 3920 3930 3940 3950 3960

AAATGAGAAT AGAAATGATT TTTGTCATCT TCTTCATTGC TGTTACCTTC ATTTCCATTT
 TTTACTCTTA TCTTTACTAA AAACAGTAGA AGAAGTAACG ACAATGGAAG TAAAGGTAAA
 Q M R I E M I F V I F F I A V T F I S I>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3440i___123 TO 4622 OF HUMAN CFTR CDNA___3480i___3490>

3970 3980 3990 4000 4010 4020

TAACAACAGG AGAAGGAGAA GGAAGAGTTG GTATTATCCT GACTTTAGCC ATGAATATCA
 ATTGTGTGCC TCTTCTCTT CCTTCTCAAC CATAATAGGA CTGAAATCGG TACTTATAGT
 L T T G E G E G R V G I I L T L A M N I>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3500i___123 TO 4622 OF HUMAN CFTR CDNA___3540i___3550>

4030 4040 4050 4060 4070 4080

TGAGTACATT GCAGTGGGCT GTAAACTCCA GCATAGATGT GGATAGCTTG ATGCGATCTG
 ACTCATGTAA CGTCACCCGA CATTGAGGT CGTATCTACA CCTATCGAAC TACGCTAGAC
 M S T L Q W A V N S S I D V D S L M R S>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3560i___123 TO 4622 OF HUMAN CFTR CDNA___3600i___3610>

4090 4100 4110 4120 4130 4140

TGAGCCGAGT CTTTAAGTTC ATTGACATGC CAACAGAAGG TAAACCTACC AAGTCAACCA
 ACTCGGCTCA GAAATTCAAG TAACGTGACG GTTGTCTTCC ATTTGGATGG TTCAGTTGGT
 V S R V F K F I D M P T E G K P T K S T>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3620i___123 TO 4622 OF HUMAN CFTR CDNA___3660i___3670>

4150 4160 4170 4180 4190 4200

AACCATACAA GAATGGCCAA CTCTCGAAAG TTATGATTAT TGAGAATTCA CACGTGAACA
 TTGGTATGTT CTTACCGGTT GAGAGCTTTC AATACTAATA ACTCTTAAGT GTGCACTTCT
 K P Y K N G Q L S K V M I I E N S H V K>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3680i___123 TO 4622 OF HUMAN CFTR CDNA___3720i___3730>

4210 4220 4230 4240 4250 4260

AAGATGACAT CTGGCCCTCA GGGGGCCAAA TGACTGTCAA AGATCTCACA GCAAAATACA
 TTCTACTGTA GACCGGAGT CCCCCGTTT ACTGACAGTT TCTAGAGTGT CGTTTATGT
 K D D I W P S G G Q H T V K D L T A K Y>

___CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON___>
 ___h___HYBRID ELA-CFTR-ELB MESSAGE___h___>
 ___3740i___123 TO 4622 OF HUMAN CFTR CDNA___3780i___3790>

4270 4280 4290 4300 4310 4320

CAGAAGGTGG AAATGCCATA TTAGAGARCA TTTCTTCTC AATAAGTCCT GGCAGAGCC
 GTCTTCCACC TTTACGGTAT AATCTCTTGT AAAGGAAGAG TTATTCAGGA CCGCTCTCCC

2145641

-77-

T E G G N A I L E N I S F S I S P G Q R >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____3800i____123 TO 4622 OF HUMAN CFTR CDNA____3840i____3850>
 4330 4340 4350 4360 4370 4380
 TGGGCCTCTT GGAAGAAGT GGATCAGGA AGAGTACTTT GTTATCAGCT TTTTGTGAGAC
 ACCCGGAGAA CCCTTCTTGA CCTAGTCCCT TCTCATGAAA CAATAGTCGA AAAAAGTCTG
 V G L L G R T G S G K S T L L S A F L R >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____3860i____123 TO 4622 OF HUMAN CFTR CDNA____3900i____3910>
 4390 4400 4410 4420 4430 4440
 TACTGAACAC TGAAGGAGAA ATCCAGATCG ATGGTGTGTC TTGGGATTCA ATAACITTTGC
 ATGACTTGTG ACTTCCTCTT TAGGTCTAGC TACCACACAG AACCTTAAGT TATTGAAACG
 L L N T E G E I Q I D G V S W D S I T L >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____3920i____123 TO 4622 OF HUMAN CFTR CDNA____3960i____3970>
 4450 4460 4470 4480 4490 4500
 AACAGTGGAG GAAAGCCTTT GGAGTGATAC CACAGAAAGT ATTTATTTTT TCTGGAACAT
 TTGTCACCTC CTTTCGGAAA CCTCACTATG GTGCTTTCA TAAATAAAAA AGACCTTGTA
 Q Q W R K A F G V I P Q K V F I F S G T >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____3980i____123 TO 4622 OF HUMAN CFTR CDNA____4020i____4030>
 4510 4520 4530 4540 4550 4560
 TTAGAAAAAA CTTGGATCCC TATGAACAGT GGAGTGATCA AGAAATATGG AAAGTTGCAG
 AATCTTTTTT GAACCTAGGG ATACTTGTC CCTCACTAGT TCTTTATACC TTTCAACGTC
 F R K N L D P Y E Q W S D Q E I W K V A >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____4040i____123 TO 4622 OF HUMAN CFTR CDNA____4080i____4090>
 4570 4580 4590 4600 4610 4620
 ATGAGGTTGG GCTCAGATCT GTGATAGAAC AGTTTCCTGG GAAGCTTGAC TTTGTCTTGC
 TACTCCAACC CGAGTCTAGA CACTATCTTG TCAAAGGACC CTTCGAACTG AAACAGGAAC
 D E V G L R S V I E Q F P G K L D F V L >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____4100i____123 TO 4622 OF HUMAN CFTR CDNA____4140i____4150>
 4630 4640 4650 4660 4670 4680
 TGGATGGGGG CTGTGTCCTA AGCCATGGCC ACAAGCAGTT GATGTGCTTG GCTAGATCTG
 ACCTACCCCC GACACAGGAT TCGGTACCCG TGTTGCTCAA CTACACGAAC CGATCTAGAC
 V D G G C V L S H G H K Q L M C L A R S >
 ____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
 ____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
 ____4160i____123 TO 4622 OF HUMAN CFTR CDNA____4200i____4210>
 4690 4700 4710 4720 4730 4742
 TTCTCAGTAA GCGGAAGATO TTGCTGCTTC ATCAACCCAG TGCTCAPTTG CATCCACTAA

AAGAGTCATT CCGTTCTAG AACGACGAAC TACTTGGGTC ACGAGTAAAC CTAGGTCATT
V L S K A K I L L L D E P S A H L D P V>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4220i____123 TO 4622 OF HUMAN CFTR CDNA____4260i____4270>

4750 4760 4770 4780 4790 4800

CATACCAAAT AATTAGAAGA ACTCTAAAC AAGCATTTGC TGATTGCACA GTAATTCTCT
GTATGGTTTA TTAATCTTCT TGAGATTTTG TTCGTAAACG ACTAACGTGT CATTAAGAGA
T Y Q I I R R T L K Q A F A D C T V I L>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4280i____123 TO 4622 OF HUMAN CFTR CDNA____4320i____4330>

4810 4820 4830 4840 4850 4860

GTGAACACAG GATAGAAGCA ATGCTGGAAT GCCAACAATT TTTGGTCATA GAAGAGAACA
CACTTGTGTC CTATCTTCGT TACGACCTTA CGGTTGTAA AAACCAGTAT CTCTCTTGT
C E H R I E A M L E C Q Q F L V I E E N>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4340i____123 TO 4622 OF HUMAN CFTR CDNA____4380i____4390>

4870 4880 4890 4900 4910 4920

AAGTGCGGCA GTACGATTCC ATCCAGAAAC TGCTGAACGA GAGGAGCCTC TTCCGGCAAG
TTCACGCCGT CATGCTAAGG TAGGCTTTTG ACGACTTGCT CTCCTCGGAG AAGGCCGTTT
K V R Q Y D S I Q K L L N E R S L F R Q>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4400i____123 TO 4622 OF HUMAN CFTR CDNA____4440i____4450>

4930 4940 4950 4960 4970 4980

CCATCAGCCC CTCCGACAGG GTGAAGCTCT TTCCCCACCG GAAGTCAAGC AAGTGCAAGT
GGTAGTCGGG GAGCTGTCC CACTTCGAGA AAGGGGTGGC CTTGAGTTTCG TTCACGTTCA
A I S P S D R V K L F P H R N S S K C K>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4460i____123 TO 4622 OF HUMAN CFTR CDNA____4500i____4510>

4990 5000 5010 5020 5030 5040

CTAAGCCCCA GATTGCTGCT CTGAAGAGG AGACAGAAGA AGAGGTGCAA GATACAAGGC
GATTGCGGGT CTAACGACGA GACTTTCTCC TCTGTCTTCT TCTCCAGTT CTATGTTCCG
S K P Q I A A L K E E T E E E V Q D T P>
____CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR; CODON____>
____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4520i____123 TO 4622 OF HUMAN CFTR CDNA____4560i____4570>

5050 5060 5070 5080 5090 5100

TTTAGAGAGC AGCATAAATG TTGACATGGG ACATTTGCTC ATGGAATTGG AGGTAGCGGA
AAATCTCTCG TCGTATTTAC AACTGTACCC TGTAACGAG TACCTTAACC TCCATCGCCT
L >

____h____HYBRID ELA-CFTR-ELB MESSAGE____h____>
____4580i____123 TO 4622 OF HUMAN CFTR CDNA____4520i____>

5110 5120 5130 5140 5150 5160
 TTGAGGTA CT GAAATGTGTG GCGGTGGCTT AAGGGTGGGA AAGAATATAT AAGGTGGGGG
 AACTCCATGA CTTTACACAC CCGCACCGAA TTCCACCCCT TTCTTATATA TTCCACCCCC
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____10_g_____ ELB 3' UNTRANSLATED SEQUENCES _____50_g_____60_____>
 _____k_____10_____k_____ ELB 3' INTRON _____k_____40_____k_____50_____>

5170 5180 5190 5200 5210 5220
 TCTCATGTAG TTTTGTATCT GTTTTGCAGC AGCCGCCGCC ATGAGCGCCA ACTCGTTTGA
 AGAGTACATC AAAACATAGA CAAAACGTCT TCGCGCGCGG TACTCGCGGT TGAGCAAAC
 _____M_____S_____A_____N_____S_____F_____D_____>
 _____IX PROTEIN (HE_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____1_____1_____1_____ IX MRNA _____1_____1_____>
 _____70_g_____ ELB 3' UNTRANSLATED SEQUENCES _____110_g_____120_____>
 _____60_____ ELB 3' INTRON _____80_____>

5230 5240 5250 5260 5270 5280
 TGGAAGCATT GTGAGCTCAT ATTTGACAAC GCGCATGCCC CCATGGGCGG GGGTGGGTCA
 ACCTTCGTAA CACTCGAGTA TAAACTGTTG CCGGTACGGG GGTACCCGGC CCCACGCACT
 G S I V S S Y L T T R M P P W A G V R Q>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); CODON_START=1_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____1_____1_____1_____ IX MRNA _____1_____1_____>
 _____130_g_____ ELB 3' UNTRANSLATED SEQUENCES _____170_g_____180_____>

5290 5300 5310 5320 5330 5340
 GAATGTGATG GGCTCCAGCA TTGATGGTCG CCCCCTCCTG CCGCAAACCT CTACTACCTT
 CTTACACTAC CCGAGGTCGT AACTACCAGC GGGGCAGGAC GGGCGTTTGA GATGATGGAA
 N V M G S S I D G R P V L P A N S T T L>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); CODON_START=1_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____1_____1_____1_____ IX MRNA _____1_____1_____>
 _____190_g_____ ELB 3' UNTRANSLATED SEQUENCES _____230_g_____240_____>

5350 5360 5370 5380 5390 5400
 GACCTACGAG ACCGTGTCTG GAACGCCGTT GGAGACTGCA GCCTCCGCCG CCGCTTCAGC
 CTGGATGCTC TGGCACAGAC CTTGCGGCAA CTTCTGACGT CGGAGGCGGC GCGCAAGTCG
 T Y E T V S G T P L E T A A S A A A S A>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); CODON_START=1_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____1_____1_____1_____ IX MRNA _____1_____1_____>
 _____250_g_____ ELB 3' UNTRANSLATED SEQUENCES _____290_g_____300_____>

5410 5420 5430 5440 5450 5460
 CGCTGCAGCC ACCGCCCCCG GGATTGTGAC TGACTTTGCT TTCCTGAGCC CGCTTGCAAG
 CGGACGTCGG TGGCGGCGC CTTACACTG ACTGAAACGA AAGGACTCGG GCGAACGTTT
 A A A T A R G I V T D F A F L S P L A S>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); CODON_START=1_____>
 _____h_____ HYBRID ELA-CFTR-ELB MESSAGE _____h_____>
 _____1_____1_____1_____ IX MRNA _____1_____1_____>
 _____310_g_____ ELB 3' UNTRANSLATED SEQUENCES _____350_g_____360_____>

5470 5480 5490 5500 5510 5520
 CAGTGACGCT TCCCGTTTAT CCGCCCCGCA TGACAAGTTG ACGGCTCTTT TGGCACAATT

GTCACGTCGA AGGGCAAGTA GCGGGGCGCT ACTGTTCAAC TGCCGAGAAA ACCGTGTTAA
 S A A S R S S A R D D K L T A L L A Q L>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); CODON_START=1____>
 _____h_____HYBRID E1A-CFTR-E1B MESSAGE_____h____>
 _____1_____1_____IX MRNA_____1_____1____>
 _____370_g_____E1B 3' UNTRANSLATED SEQUENCES_____410_g_____420____>

5530 5540 5550 5560 5570 5580
 GGATTCTTTG ACCCGGGAAC TTAATGTCGT TTCTCAGCAG CTGTTGGATC TGCGCCAGCA
 CCTAAGAAAC TGGGCCCTTG AATTACAGCA AAGAGTCGTC GACAACCTAG ACGCGGTCGT
 D S L T R E L N V V S Q Q L L D L R Q Q>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); CODON_START=1____>
 _____h_____HYBRID E1A-CFTR-E1B MESSAGE_____h____>
 _____1_____1_____IX MRNA_____1_____1____>
 _____430_g_____E1B 3' UNTRANSLATED SEQUENCES_____470_g_____480____>

5590 5600 5610 5620 5630
 GGTTTCTGCC CTGAAGGCTT CCTCCCTCC CAATGCGGTT TAAACATAA ATAAA
 CCAAAGACGG GACTTCCGAA GGAGGGGAGG GTTACGCCAA ATTTTGTATT TATTT
 V S A L K A S S P P N A V *>
 _____IX PROTEIN (HEXON-ASSOCIATED PROTEIN); C_____>
 _____h_____HYBRID E1A-CFTR-E1B MESSAGE_____h____>
 _____1_____1_____IX MRNA_____1_____1____>
 _____490_g_____E1B 3' UNTRANSLATED SEQUENCES_____530_g_____>

Table III

Nucleotide Sequence Analysis of Ad2-ORF6/PGK-CFTR

| | | | |
|------------|----------------------------|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| LOCUS | AD2-ORF6/P 36335 BP DS-DNA | | |
| DEFINITION | - | | |
| ACCESSION | - | | |
| KEYWORDS | - | | |
| SOURCE | - | | |
| FEATURES | From | To/Span | Description |
| frag | 12915 | 36335 | 10676 to 34096 of Ad2-E4/ORF6 |
| frag | 35069 | 35973 | 33178 to 34082 of Ad2 seq |
| pre-msg | > 35973 | < 35069 (C) | E4 mRNA [Nucleic Acids Res. 9, 1675-1689 (1981)], [J. Mol. Biol. 149, 189-221 (1981)], [Nucleic Acids Res. 12, 3503-3519 (1984)], [Unpublished (1984)] [Split] |
| IVS | 35794 | 35084 (C) | E4 mRNA intron D7 [J. Virol. 50, 106-117 (1984)], [Nucleic Acids Res. 12, 3503-3519 (1984)], [Unpublished (1984)] |
| IVS | 35794 | 35175 (C) | E4 mRNA intron D6 [Nucleic Acids Res. 12, 3503-3519 (1984)] |
| IVS | 35794 | 35268 (C) | E4 mRNA intron D5 [J. Virol. 50, 106-117 (1984)] |
| IVS | 35794 | 35295 (C) | E4 mRNA intron D4 [J. Virol. 50, 106-117 (1984)] |
| IVS | 35794 | 35343 (C) | E4 mRNA intron D3 [J. Virol. 50, 106-117 (1984)] |
| IVS | 35794 | 35501 (C) | E4 mRNA intron D2 [J. Virol. 50, 106-117 (1984)] |
| IVS | 35794 | 35570 (C) | E4 mRNA intron D1 [J. Virol. 50, 106-117 (1984)] |
| IVS | 35794 | 35766 (C) | E4 mRNA intron D [J. Virol. 50, 106-117 (1984)] |
| frag | 35978 | 36335 | 35580 to 35937 of Ad2 seq |
| pre-msg | 36007 | < 35978 (C) | E4 mRNA [Nucleic Acids Res. 9, 1675-1689 (1981)], [J. Mol. Biol. 149, 189-221 (1981)], [Nucleic Acids Res. 12, 3503-3519 (1984)], [Unpublished (1984)] [Split] |
| rpt | 36234 | 36335 | inverted terminal repetition; 99.54% [Biochem. Biophys. Res. Commun. 87, 671-678 (1979)], [J. Mol. Biol. 128, 577-594 (1979)] |
| frag | ~ 12915 | 35054 | 1 to 32815 of Ad2 seq [Split] |
| pept | < 28478 | 28790 | 3 33K protein (virion morphogenesis) |
| pept | 28478 | 28790 | 1 33K protein (virion morphogenesis); codon_start=1 |
| mRNA | 29331 | < 12915 (C) | E2b mRNA [J. Biol. Chem. 257, 13475-13491 (1982)] [Split] |
| pre-msg | < 12915 | 16352 | major late mRNA L1 (alt.) [J. Mol. Biol. 149, 189-221 (1981)], [J. Virol. 48, 127-134 (1983)] [Split] |
| pre-msg | < 12915 | 20208 | major late mRNA L2 (alt.) [J. Mol. Biol. 149, 189-221 (1981)], [J. Virol. 38, 469-482 (1981)], [J. Virol. 48, 127-134 (1983)] [Split] |
| pre-msg | < 12915 | 24682 | major late mRNA L3 (alt.) [Nucleic Acids Res. 9, 1-17 (1981)], [J. Mol. Biol. 149, 189-221 (1981)], [J. Virol. 48, 127-134 (1983)] [Split] |
| pre-msg | < 12915 | 30462 | major late mRNA L4 (alt.) [J. Mol. Biol. 149, 189-221 (1981)], [J. Virol. 48, 127-134 (1983)] [Split] |
| pre-msg | < 12915 | 35037 | major late mRNA L5 (alt.) [J. Mol. Biol. 149, 189-221 (1981)], [J. Virol. 48, 127-134 (1983)] [Split] |

Nucleotide Sequence Analysis (cont.)

| | | | |
|---------|---------|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| mRNA | < 12915 | 13278 | major late mRNA intron (precedes 52,55K mRNA; 1st L1 mRNA) [Cell 16, 841-850 (1979)], [Cell 16, 851-861 (1979)], [J. Mol. Biol. 134, 143-158 (1979)], [J. Mol. Biol. 135, 413-433 (1979)], [Nature 292, 420-426 (1981)] [Split] |
| IVS | < 12915 | 16388 | major late mRNA intron (precedes penton mRNA; 1st L2 mRNA) [J. Virol. 48, 127-134 (1983)] [Split] |
| IVS | < 12915 | 18754 | major late mRNA intron (precedes pV mRNA; 2nd L2 mRNA) [J. Biol. Chem. 259, 13980-13985 (1984)] [Split] |
| FVS | < 12915 | 20238 | major late mRNA intron (precedes pVI mRNA; 1st L3 mRNA) [J. Virol. 38, 469-482 (1981)] [Split] |
| IVS | < 12915 | 21040 | major late mRNA intron (precedes hexon mRNA; 2nd L3 mRNA) [Proc. Natl. Acad. Sci. U.S.A. 75, 5822-5826 (1978)], [Cell 16, 841-850 (1979)] [Split] |
| IVS | < 12915 | 23888 | major late mRNA intron (precedes 23K mRNA; 3rd L3 mRNA) [Nucleic Acids Res. 9, 1-17 (1981)] [Split] |
| IVS | < 12915 | 26333 | major late mRNA intron (precedes 100K mRNA; 1st L4 mRNA) [Virology 128, 140-153 (1983)] [Split] |
| RNA | < 12915 | 13005 | VA I RNA (alt.) [J. Biol. Chem. 252, 9043-9046 (1977)] [Split] |
| RNA | < 12915 | 13005 | VA I RNA (alt.) [J. Biol. Chem. 246, 6991-7009 (1971)], [J. Biol. Chem. 252, 9047-9054 (1977)], [Proc. Natl. Acad. Sci. U.S.A. 77, 2424-2428 (1980)] [Split] |
| ???? | < 12915 | 13262 | VA II RNA [Proc. Natl. Acad. Sci. U.S.A. 77, 3778-3782 (1980)], [Proc. Natl. Acad. Sci. U.S.A. 77, 2424-2428 (1980)] [Split] |
| pept | 13279 | 14526 | 1 52,55K protein; codon_start=1 |
| pept | 14547 | 16304 | 1 IIIa protein (peripentonal hexon-associated protein; splice sites not sequenced); codon_start=1 |
| signal | 16331 | 16336 | major late mRNA L1 poly-A signal (putative) 39.21% |
| pept | 16390 | 18105 | 1 penton protein (virion component III); codon_start=1 |
| pept | 18112 | 18708 | 1 Pro-VII protein (precursor to major core protein); codon_start=1 |
| pept | 18778 | 19887 | 1 pV protein (minor core protein); codon_start=1 |
| signal | 20188 | 20193 | major late mRNA L2 polyadenylation signal (putative) 49.94% |
| pept | 20240 | 20992 | 1 pVI protein (hexon-associated precursor); codon_start=1 |
| pept | 21077 | 23983 | 1 hexon protein (virion component II); codon_start=1 |
| ???? | < 12915 | 24631 | 23K protein (endopeptidase); codon_start=1 [Split] |
| signal | 24657 | 24662 | major late mRNA L3 polyadenylation signal (putative); 62.38% |
| pre-msg | 28193 | 24659 (C) | E2a late mRNA (alt.) [J. Mol. Biol. 149, 189-221 (1981)] |
| pre-msg | 28195 | 24659 (C) | E2a late mRNA (alt.) [Nucleic Acids Res. 12, 3503-3519 (1984)], [Unpublished (1984)] |
| pre-msg | 29330 | 24659 (C) | E2a early mRNA (alt.) [J. Mol. Biol. 149, |

2145641

-83-

Nucleotide Sequence Analysis (cont.)

| | | | | |
|---------|---------|---------|-----|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| pre-msg | 29331 | 24659 | (C) | 189-221 (1981)] |
| signal | 24683 | 24678 | (C) | E2a early mRNA (alt.) [J. Mol. Biol. 149, 189-221 (1981)] |
| pept | 26318 | 24729 | (C) | E2a mRNA polyadenylation signal on comp strand (putative); 62.43% |
| IVS | 26953 | 26328 | (C) | DBP protein (DNA binding or 72K protein); codon_start=1 |
| pept | 26347 | 28764 | 1 | E2a mRNA intron B [Nucleic Acids Res. 9, 4439-4457 (1981)] |
| IVS | 29263 | 27031 | (C) | 100K protein (hexon assembly); codon_start=1 |
| IVS | 28124 | 27211 | (C) | E2a early mRNA intron A [Cell 18, 569-580 (1979)] |
| IVS | 28791 | 28992 | | E2a late mRNA intron A [Virology 128, 140-153 (1983)] |
| pept | 28993 | > 29366 | 1 | 33K-pept intron [J. Virol. 45, 251-263 (1983)] |
| pept | 29454 | 30137 | 1 | 33K protein (virion morphogenesis) |
| mRNA | 29848 | 33103 | | pVIII protein (hexon-associated precursor); codon_start=1 |
| IVS | 30220 | 30614 | | E3-2 mRNA; 85.88% [Gene 22, 157-165 (1983)] |
| signal | 30444 | 30449 | | major late mRNA intron ('x' leader) [Gene 22, 157-165 (1983)], [J. Biol. Chem. 259, 13980-13985 (1984)] |
| signal | < 12915 | 32676 | | major late mRNA L4 polyadenylation signal; (putative) 78.48% |
| pept | 31051 | 31530 | 1 | major late mRNA intron ('y' leader) [J. Mol. Biol. 135, 413-433 (1979)], [J. Virol. 38, 469-482 (1981)], [EMBO J. 1, 249-254 (1982)], [Gene 22, 157-165 (1983)] [Split] |
| pept | 31707 | 32012 | 1 | E3 19K protein (glycosylated membrane protein); codon_start=1 |
| signal | 32008 | 32013 | | E3 11.6K protein; codon_start=1 |
| IVS | 32822 | 33268 | | E3-1 mRNA polyadenylation signal (putative); 82.69% |
| signal | 33081 | 33086 | | major late mRNA intron ('z' leader) [Proc. Natl. Acad. Sci. U.S.A. 75, 5822-5826 (1978)], [Cell 16, 841-850 (1979)], [EMBO J. 1, 249-254 (1982)], [Gene 22, 157-165 (1983)] |
| ???? | < 12915 | 35017 | | E3-2 mRNA polyadenylation signal; 85.82% (putative) |
| signal | 35013 | 35018 | | fiber protein (virion component IV); codon_start=1 [Split] |
| pre-msg | 35054 | > 35041 | (C) | major late mRNA L5 polyadenylation signal; (putative) 91.19% |
| frag | 1 | 12914 | | E4 mRNA [Nucleic Acids Res. 9, 1675-1689 (1981)], [J. Mol. Biol. 149, 189-221 (1981)], [Nucleic Acids Res. 12, 3503-3519 (1984)], [Unpublished (1984)] [Split] |
| DNA | 1 | > 356 | | 1 to 12914 of pAd2/PCR-CFTR |
| rpt | 1 | > 103 | | 1 to 357 Ad2 |
| | < 10 | 103 | | inverted terminal repetition; 0.28% [Biochem. Biophys. Res. Commun. 87, 671-678 (1979)], [J. Mol. Biol. 128, 577-594 (1979)] |
| frag | 357 | 379 | | inverted terminal repetition; 0.28% [Biochem. Biophys. Res. Commun. 87, 671-678 (1979)], [J. Mol. Biol. 128, 577-594 (1979)] [Split] |
| frag | 915 | > 923 | | linker segment |
| | | | | polylinker cloning sites [Split] |

-84-

Nucleotide Sequence Analysis (cont.)

| | | |
|------------|--------------------------------------|---------------------------------------------------------------------------------------------------------------|
| DNA | < 924 > 954 | polylinker cloning sites [Split] |
| signal | < 5567 > 12914 | 3328 to 10685 of Ad2 [Split] |
| frag | 380 914 | pgk promoter |
| | < 955 > 958 | polylinker cloning sites [Split] |
| signal | < 5501 5522 | polylinker cloning sites [Split] |
| frag | 5523 5555 | syn. BGH poly A |
| | < 5564 5567 | linker [Split] |
| frag | 5555 > 5560 | linker [Split] |
| revision | 5564 5567 | 920 to 5461 of pCMV-CFTR-936C |
| | 559 5500 | mistake in published sequence of Riordan et al. C not A is correct = N to H a.a. change |
| modified | 2868 2868 | 936 T to C mutation to inactivate cryptic bacterial promoter. Silent amino acid change |
| site | < 959 975 | polylinker segment from pCMV-CFTR-936C (Rc/CMV-Invitrogen SpeI-BstXI) [Split] |
| site | 976 990 | linker segment from pCMV-CFTR-936C. Originally SalI/BstXI adaptor oligo 1499DS |
| site | 991 1001 | linker segment from pCMV-CFTR-936C. Originally from pMT-CFTR construction oligo 1247 RG -Sal I to AvaI sites. |
| mRNA | 1001 > 5500 | 123 to 4622 of HUMCFTR |
| pept | 1011 > 5453 | 1 cystic fibrosis transmembrane conductance regulator; codon_start=1 |
| BASE COUNT | 8597 A 10000 C 9786 G 7952 T 0 OTHER | |
| ORIGIN | ? | |

Ad2-ORF6/P Length: 36335 Sep 16, 1993 - 08:13 PM Check: 1664 ..

```

1 CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGCACT
61 TTGTGACGTG GCGCGGGGCG TCGGAACGGG GCGGGTGAAG TAGTAGTGTG GCGGAAGTGT
121 GATGTTGCAA GTGTGGGGGA ACACATGTAA GCGCCGGATG TGGTAAAAGT GACGTTTGTG
181 GTGTGGGCGG GTGTATACGG GAAGTGACAA TTTTCGGCGG GTTTTAGGCG GATGTTGTAG
241 TAAATTGCGG CGTAACCAAG TAATGTTTGG CCATTTTCGC GGGAAAACCTG AATAAGAGGA
301 AGTGAAATCT GAATAATTCT GTGTTACTCA TAGCGGTAA TATTTGTCTA GGGCCGCTCG
361 AGGTGACCGG TCTATCGATA AGCTTGATAT CGAATTCGGG GGTTCGGGTT GCGCCTTTTC
421 CAAGGCAGCC CTGGGTTTGC GCAGGGAGCG GGCTGCTCTG GGCGTGGTTC CGGGAACGCG
481 AGCGGGGCGG ACCCTGGGTC TCGCACATTC TTCACGTCCG TTCGCAGCGT CACCCGAGTC
541 TTCCGCGCTA CCCTTGTGGG CCCCCGGGCG ACGCTTCTC GTCCGCCCCCT AAGTCGGGAA
601 GGTTCCTTGC GGTTCGCGGC GTGCCGGACG TGACAAACGG AAGCGGCACG TCTCACTAGT
661 ACCCTCGCAG ACGGACAGCG CCAGGGAGCA ATGGCAGCGC GCGGACCGCG ATGGGCTGTG
721 GCCAATAGCG GCTGCTCAGC AGCGCGCGCC GAGACGAGCG GCGGGGAAGG GGCGGTGCGG
781 GAGGCGGGGT GTGGGGCGGT AGTGTGGGCC CTGTTCCCTG CCGCGCGGTG TTCCGCATTC
841 TGCAAGCCTC CGGAGCGCAC GTGGGCAGTC GGCTCCCTCG TTGACGGAAT CACCGACCTC
901 TCTCCCCAGG ATCCACTAGT ATTAAATCGT ACGCCTAGTA TTTAAATCGT ACGCCTAGTA
961 ACGGCCGCCA GTGTGCTGCA GATATCAAAG TCGACCGTAC CCGAGAGACC ATGCAGAGGT
1021 CGCCTCTGGA AAAGGCCAGC GTTGTCTCCA AACTTTTTTT CAGCTGGACC AGACCAATTT
1081 TGAGGAAAGG ATACAGACAG CGCCTGGAAT TGTACAGCAT ATACCAAATC CCTTCTGTTG
1141 ATTCTGCTGA CAATCTATCT CAAAAATTGG AAAGAGAATG GGATAGAGAG CTGGCTTCAA
1201 AGAAAAATCC TAAACTCATT AATGCCCTTC GCGGATGTTT TTTCTGGAGA TTTATGTTCT
1261 ATGGAATCTT TTTATATTTA GGGGAAGTCA CCAAAGCAGT ACAGCCTCTC TTAGTGGGAA
1321 GAATCATAGC TTCCTATGAC CCGGATAACA AGGAGGAACG CTCTATCGCG ATTTATCTAG
1381 GCATAGGCTT ATGCCTTCTC TTTATTGTGA GGACACTGCT CCTACACCCA GGCATTTTGT
1441 GCCTTCATCA CATTGGAATG CAGATGAGAA TAGCTATGTT TAGTTTGATT TATAAGAAGA
1501 CTTTAAAGCT GTCAAGCCGT GTTCTAGATA AAATAAGTAT TGGACAACTT GTTAGTCTCC
1561 TTTCCAACAA CCTGAACAAA TTTGATGAAG GACTTGCAAT GGCACATTTT GTGTGGATCG
1621 CTCCTTTGCA AGTGGCACTC CTCATGCGGC TAATCTGGGA GTTGTTACAG GCGTCTGCCT
1681 TCTGTGGACT TGGTTTCTCT ATAGTCCTTG CCCTTTTTTCA GGCTGGGCTA GGGAGAATGA
1741 TGATGAAGTA CAGAGATCAG AGAGCTGGGA AGACTCAGTA AAGACTTTGT ATTACCTCAG
1801 AAATGATTGA AAACATCCAA TCTGTTAAGG CATACTGCTG GGAAGAAGCA ATGCAAAAAA

```


Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|------|-------------|------------|-------------|-------------|-------------|-------------|
| 1861 | TGATTGAAAA | CTTAAGACAA | ACAGAAGCTGA | AACTGACTOG | GAAGGCAGCC | TATGTGAGAT |
| 1921 | ACTTCAATAG | CTCAGCCTTC | TTCTTCTCAG | GGTTCCTTGT | GGTGTMTTFA | TCGTGTCTTC |
| 1981 | CCTATGCACT | AATCAAAGGA | ATCATCCTCC | GGAAAAATATT | CACCAACCATC | TCATTCTGCA |
| 2041 | TTGTTCCTGG | CATGGGCGTC | ACTGGGCAAT | TTCCCTGGGC | TGTACAAACA | TGGTATGACT |
| 2101 | CTCTTGGAGC | AATAAACAAA | ATACAGGATT | TCTTACAAAA | GCAAGAAATAT | AAGACATTGG |
| 2161 | AATATAACTT | AACGACTACA | GAAGTAGTGA | TGGAGAATGT | AACAGCCCTTC | TGGGAGGAGG |
| 2221 | GATTTGGGGA | ATTATTTGAG | AAAGCAAAC | AAAACAATAA | CAATAGAAAA | ACTTCTAATG |
| 2281 | GTGATGACAG | CCTCTTCTTC | AGTAATTTCT | CACCTCTTGG | TACTCCTGTC | CTGAAAGATA |
| 2341 | TTAATTTCAA | GATAGAAAGA | GGACAGTTGT | TGGCGGTTGC | TGGATCCACT | GGAGCAGGCA |
| 2401 | AGACTTCAGT | TCTAATGATG | ATTATGGGAG | AACTGGAGCC | TTGAGAGGGT | AAAATTAAGC |
| 2461 | ACAGTGGGAG | AATTTTCATT | TGTTCTCAGT | TTTCTCTGGAT | TATGCCTGGC | ACCATTAAAG |
| 2521 | AAAATATCAT | CTTTGGTGT | TCCTATCATG | AATATAGATA | CAGAAGCGTC | ATCAAAGCAT |
| 2581 | GCCAACTAGA | AGAGGACATC | TCCAGTTTGG | CAGAGAAAGA | CAATATAGTT | CTTGGAGAAAG |
| 2641 | GTGGAATCAC | ACTGAGTGGG | GGTCAACGAG | CAAGAATTTT | TTTAGCAAGA | GCAGTATACA |
| 2701 | AAGATGCTGA | TTTGATTTTA | TTAGACTCTC | CTTTTGGATA | CCTAGATGTT | TTAACAGAAA |
| 2761 | AAGAAATATT | TGAAAGCTGT | GTCTGTAAAC | TGATGGCTAA | CAAAACTAGG | ATTTTGGTCA |
| 2821 | CTTCTAAAT | GGAAACATTT | AAGAAAGCTG | ACAAAATATT | AATTTTGCAT | GAAAGGTAGCA |
| 2881 | GCTATTTTFA | TGGGACATTT | TCGAACTTTC | AAAATCTACA | GCCAGACTTT | AGCTCAAAAC |
| 2941 | TCATGGGATG | TGATTCTTTC | GACCAATTTA | GTGCAGAAAG | AAGAAATTC | ATCCTAACTG |
| 3001 | AGACCTTACA | CGGTTTCTCA | TTAGAAGGAG | ATGCTCCTGT | CTCCTGGACA | GAAACAAAAA |
| 3061 | AACAACTTT | TAAACAGACT | GGAGAGTTTG | GGGAAAAAAG | GAAGAAATCT | ATCTCAATC |
| 3121 | CAATCAACTC | TATACGAAAA | TTTTCCATTG | TGCAAAAGAC | TCCCTTACAA | ATGAATGGCA |
| 3181 | TCGAGAGGGA | TTCTGATGAG | CCTTTAGAGA | GAAGGCTGTC | CTTAGTACCA | GATTCGTAGC |
| 3241 | AGGGAGAGGC | GATACTGCCT | CGCATCAGCG | TGATCAGCAC | TGGCCCCACG | CTTCAGGAC |
| 3301 | GAAGGAGGCA | GTCTGTCTCG | AACCTGATGA | CACACTCAGT | TAACCAAGGT | CAGAACATTC |
| 3361 | ACCGAAAGAC | AACAGCATCC | ACACGAAAAG | TGTCACTGGC | CCCTCAGGCA | AACCTGACTG |
| 3421 | AACCTGGATAT | ATATTCAAGA | AGGTTATCTC | AAGAAACTGG | CTTGGAAATA | AGTGAAGAAA |
| 3481 | TTAAGGAAGA | AGACTTAAAG | GAGTGCCCTT | TTGATGATAT | GGAGAGCATA | CCAGCAGTGA |
| 3541 | CTACATGGAA | CACATACCTT | OGATATATTA | CTGTCCACAA | GAGCTTAATT | TTTGTGCTAA |
| 3601 | TTTGGTGCTT | AGTAATTTTT | CTGGCAGAGG | TGCTGTCTTC | TTTGGTTGTG | CTGTGGCTCC |
| 3661 | TTGGAAACAC | TCCTCTTCAA | GACAAAGGGA | ATAGTACTCA | TAGTAGAAAT | AACAGCTATG |
| 3721 | CAGTGATTAT | CACCAGCACC | AGTTGCTATT | ATGTGTTTTA | CATTTACGTC | GGAGTAGCCG |
| 3781 | ACACTTTGCT | TGCTATGGGA | TTCTTCAGAG | GTCTACCACT | GGTGCATACT | CTAATCACAG |
| 3841 | TGTGGAATA | TTTACACCAC | AAAATGTTAC | ATTCTGTCTT | TCAAGCACCT | ATGTCAACCC |
| 3901 | TCAACACGTT | GAAAGCAGGT | GGGATTCCTA | ATAGATTCTC | CAAAGATATA | GCAATTTTGG |
| 3961 | ATGACCTTCT | GCCTCTTACC | ATATTGACT | TCATCCAGTT | GTTATTAAAT | GTGATTGGAG |
| 4021 | CTATAGCAGT | TGTGCGCAGT | TTACAACCCCT | ACATCTTTGT | TGCAACAGTG | CCAGTGATAG |
| 4081 | TGGCTTTTAT | TATGTTGAGA | GCATATTTCC | TCCAAACCTC | ACAGCAACTC | AAACAACTGG |
| 4141 | AATCTGAAGG | CAGGAGTCCA | ATTTTCACTC | ATCTTGTTAC | AAGCTTAAAA | GGACTATGGA |
| 4201 | CACCTTCTGC | CTTCGGACGG | CAGCCTTACT | TTGAAACTCT | GTTCCACAAA | GCTCTGAATT |
| 4261 | TACATACTGC | CAACTGGTTC | TTGTACCTGT | CAACACTGCG | CTGGTTCCAA | ATGAGAATAG |
| 4321 | AAATGATTTT | TGTCATCTTC | TTTATTGCTG | TTACCTTCAT | TTCCATTTTA | ACAACAGGAG |
| 4381 | AAGGAGAAGG | AAGAGTTGGT | ATTATCCTGA | CTTTAGCCAT | GAATATCATG | AGTACATTGC |
| 4441 | AGTGGGCTGT | AAACTCCAGC | ATAGATCTGG | ATAGCTTGAT | GOGATCTGTG | AGCCGAGTCT |
| 4501 | TTAAGTTTCAT | TGACATGCCA | ACAGAAGGTA | AACCTACCAA | GTCAACCAAA | CCATACAAGA |
| 4561 | ATGGCCAACT | CTCGAAAGTT | ATGATTATTG | ACAATTCACA | CGTGAAGAAA | GATGACATCT |
| 4621 | GGCCCTCAGG | GGGCCAAATG | ACTGTCAAAG | ATCTCACAGC | AAAATACACA | GAAGGTGGAA |
| 4681 | ATGCCATATT | AGAGAACATT | TCCTTCTCAA | TAAGTCTCTG | CCAGAGGGTG | GGCCTCTTGG |
| 4741 | GAAGAACTGG | ATCAGGGAAG | AGTACTTTGT | TATCAGCTTT | TTTGAGACTA | CTGAACACTG |
| 4801 | AAGGAGAAAT | CCAGATCGAT | GGTGTGTCTT | CGGATTCAAT | AACTTTGCAA | CAGTGGAGGA |
| 4861 | AAGCCTTTGG | AGTGATACCA | CAGAAAGTAT | TTATTTTTC | TGGAACATT | AGAAAAAAT |
| 4921 | TGGATCCCTA | TGAACAGTGG | AGTGATCAA | AAATATGGAA | AGTTGCAGAT | GAGGTTGGCC |
| 4981 | TCAGATCTGT | GATAGAACAG | TTTCTCTGGA | AGCTTGACTT | TGTCCTTGTG | GATGGGGGCT |
| 5041 | GTGTCTTAAG | CCATGGCCAC | AAGCAGTTGA | TGTGCTTGGC | TAGATCTGTT | CTCAGTAAGG |
| 5101 | CGAAGATCTT | GCTGCTTGAT | GAACCCAGTG | CTCATTTGGA | TCCAGTAACA | TACCAATAA |
| 5161 | TTAGAAGAAC | TCTAAAACAA | GCATTTGCTG | ATTGCACAGT | AATTCTCTGT | GAACACAGGA |
| 5221 | TAGAAGCAAT | GCTGGAATGC | CAACAATTTT | TGTCATAGA | AGAGAACAAA | GTGCGGCAGT |

Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|------|-------------|-------------|-------------|------------|------------|-------------|
| 5281 | ACGATTCCAT | CCAGAAACTG | CTGAACGAGA | GGAGCCTCTT | COGGCAAGCC | ATCAGCCCOCT |
| 5341 | CCGACAGGGT | GAAGCTCTTT | CCCCACCGEA | ACTCAAGCAA | GTGCAAGTCT | AAGCCOCAGA |
| 5401 | TTGCTGCTCT | GAAACAGGAG | ACAGAAGAAG | AGGTGCAAGA | TACAAGGCCT | TAGAGAGCAG |
| 5461 | CATAAATGTT | GACATGGGAC | ATTTGCTCAT | OGAATTGGAG | AAATCGTACG | CTTAGGACCG |
| 5521 | GTAATAAAAT | GAGGAAATTG | CATCGCATTG | TCGACCGCGT | TACGCGGGAA | GGTCTGAGG |
| 5581 | TACGATGAGA | CCCCCAACCAG | GTGCAGACCC | TGCGAGTGTG | GCGGTAAACA | TATTAGGAAC |
| 5641 | CAGCCTGTGA | TGCTGGATGT | GACCGAGGAG | CTGAGGCCCG | ATCACTTGGT | GCTGGCCTGC |
| 5701 | ACCCGCGCTG | AGTTTGGCTC | TAGCGATGAA | GATACAGATT | GAGGTACTGA | AAATGCTGGG |
| 5761 | CGTGGCTTAA | GGGTGGGAAA | GAATATATAA | GGTGGGGGTC | TCATGTAGTT | TTGTATCTGT |
| 5821 | TTTGCAGCAG | COGCGCCCAT | GAGCGCCAAC | TGGTTTGATG | GAAGCATTGT | GAGCTCATAT |
| 5881 | TTGACAACGC | GCATGCCCCC | ATGGGCGGGG | GTGCGTCAGA | ATGTGATGGG | CTCCAGCATT |
| 5941 | GATGGTGGCC | CCGTCTGGCC | CGCAAACTCT | ACTACCTTGA | CCTACGAGAC | CGTGTCTGGA |
| 6001 | ACGCGGTTGG | AGACTGCAGC | CTCGCGCGCC | GCTTCAGCCG | CTGCAGCCAC | CGCCCGCGGG |
| 6061 | ATTGTGACTG | ACTTTGCTTT | CCTGAGCCCG | CTTGCAAGCA | GTGCAGCTTC | CCGTTTCATCC |
| 6121 | GCCCGCGATG | ACAAGTTGAC | GGCTCTTTTG | GCACAATTGG | ATTCTTTGAC | COGGGAACCT |
| 6181 | AATGTGTTTT | CTCAGCAGCT | GTTGGATCTG | CGCCAGCAGG | TTTCTGCCCT | GAAGGCTTCC |
| 6241 | TCCCCGCCCA | ATGCGGTTTA | AAACATAAAT | AAAAACCAGA | CTCTGTTTGG | ATTTTGATCA |
| 6301 | AGCAAGTCTC | TTCCTGTCTT | TATTTAGGGG | TTTTGCGGCG | GCGGTAGGCC | CGGGACCAGC |
| 6361 | GGTCTCGGTC | GTTGAGGGTC | CTGTGTATTT | TTTCCAGGAC | GTGGTAAAGG | TGACTCTGGA |
| 6421 | TGTTTCAGATA | CATGGGCATA | AGCCCGTCTC | TGGGGTGGAG | GTAGCACCAC | TGCAGAGCTT |
| 6481 | CATGCTGCGG | GGTGGTGTG | TAGATGATCC | AGTCGTAGCA | GGAGCGCTGG | GGTGGTGGCC |
| 6541 | TAAAAATGTC | TTTCAGTAGC | AAGCTGATTG | CCAGGGGCAG | GCCCTTGGTG | TAAGTGTTTA |
| 6601 | CAAAGCGGTT | AAGCTGGGAT | GGGTGCATAC | GTOGGGATAT | GAGATGCATC | TTGGACTGTA |
| 6661 | TTTTTAGGTT | GGCTATGTTT | CCAGCCATAT | CCCTCCGGGG | ATTCAATGTT | TGCAGAACCA |
| 6721 | CCAGCACAGT | GTATCCGGTG | CACTTGGGAA | ATTTGTCTAT | TAGCTTAGAA | GGAAATGOGT |
| 6781 | GGAAGAACCT | GGAGACGCCC | TTGTGACCTC | CGAGATTTTC | CATGCATTCC | TCCATAATGA |
| 6841 | TGGCAATGGG | CCCACGGGCG | GCGGCTCTGG | CGAAGATATT | TCGCGGATCA | CTAACCTCAT |
| 6901 | AGTTGTGTTT | CAGGATGAGA | TCGTCTATAG | CCATTTTAC | AAAGCGCGGG | CGGAGGGTGC |
| 6961 | CAGACTGCGG | TATAATGGTT | CCATCCGGCC | CAGGGGCGTA | GTTACCCCTA | CAGATTTGCA |
| 7021 | TTTCCCAACG | TTTGAGTTCA | GATGGGGGGA | TCATGTCTAC | CTGCGGGGCG | ATGAAGAAAA |
| 7081 | CCGTTTCCGG | GGTAGGGGAG | ATCAGCTGGG | AAGAAAGCAG | GTTCCTGAGC | AGCTGCGACT |
| 7141 | TACCGCAGCC | GGTGGGCCCC | TAAATCACAC | CTATTACCGG | CTGCAACTGG | TAGTTAAGAG |
| 7201 | AGCTGCAGCT | GCCGTCAATC | CTGAGCATTG | GGGCCACTTC | GTTAAGCATG | TCCCTGACTT |
| 7261 | GCAATGTTTT | CCTGACCAAA | TGCGCCAGAA | GGCGCTCGCC | GCCCAGCGAT | AGCAGTTCTT |
| 7321 | GCAAGGAAGC | AAAGTTTTTC | AACGGTTTTG | GGCGTCCGCG | CGTAGGCATG | CTTTTGAGCG |
| 7381 | TTTGACCAAG | CAGTTCCAGG | CGGTCCACAC | GCTCGGTAC | GTGCTCTAAG | GCATCTCGAT |
| 7441 | CCAGCATATC | TCCTCGTTTT | GCGGGTTGGG | GCGGCTTTCC | CTGTACGGCA | GTAGTCCGGT |
| 7501 | CTCGTCCAGA | CGGGCCAGGG | TCATGTCTTT | CCACGGGCGC | AGGGTCCCTG | TCAGCGTAGT |
| 7561 | CTGGGTACAG | GTGAAGCGGT | GCGCTCCGGG | CTGCGCGCTG | GCCAGGGTGC | GCTTGAGGCT |
| 7621 | GGTCTGTCTG | GTGCTGAAGC | GCTGCCGGTC | TTGCGCCCTG | GCGTGGGCCA | GGTAGCATTT |
| 7681 | GACCATGGTG | TCATAGTCCA | GCCCCTCCGC | GGCGTGGCCC | TTGGCGCGCA | GCTTGCCCTT |
| 7741 | GGAGGAGGGG | CCGCACGAGG | GGCAGTGCAG | ACTTTTAAGG | GCGTAGAGCT | TGGGCGCGAG |
| 7801 | AAATACCGAT | TCCGGGGAGT | AGGCATCCCG | GCGCAGGCCC | CCGCAGACGG | TCTCGCATTC |
| 7861 | CACGAGCCAG | GTGAGCTCTG | GCCGTTCCGG | GTCAAAAACC | AGGTTTCCCC | CATGCTTTTT |
| 7921 | GATGCGTTTT | TTACCTCTGG | TTTCATGAG | CCGGTGTTCA | CGCTCGGTGA | CGAAAAGGCT |
| 7981 | GTCCGTGTCC | CCGTATACAG | ACTTGAGAGG | CCTGTCTCTG | AGCGGTGTTT | CGCGGTCTCT |
| 8041 | CTCGTATAGA | AACTCGGACC | ACTCTGAGAC | GAAGGCTCGG | GTCCAGGCCA | GCACGAAGGA |
| 8101 | GGCTAAGTGG | GAGGGGTAGC | GGTCGTGTGC | CACTAGGGGG | TCCACTCGCT | CCAGGGTGTG |
| 8161 | AAGACACATG | TGCGCCTCTT | CGCATCAAG | GAAGGTGATT | GGTTTATAGG | TGTAGGCCAC |
| 8221 | GTGACCGGGT | GTTCTCTGAAG | GGGGGCTATA | AAAGGGGGTG | GGGGCGCGTT | CGTCTCTACT |
| 8281 | CTCTTCCGCA | TCGCTGTCTG | CGAGGGCCAG | CTGTTGGGGT | GAGTACTCCC | TCTCAAAAGC |
| 8341 | GGGCATGACT | TCTGCGCTAA | GATTGTTCAGT | TTCCAAAAAC | GAGGAGGATT | TGATATTAC |
| 8401 | CTGGCCCGCG | GTGATGCCCT | TGAGGGTGGC | CGCGTCCATC | TGGTCAGAAA | AGACAATCTT |
| 8461 | TTTGTGTGTA | AGCTTGGTGG | CAAAACGACC | GTAGAGGGCG | TTGGACAGCA | ACTTGGCGAT |
| 8521 | GGAGCGCAGG | GTTTGGTTTT | TGTCGGGATC | GGCGCGCTCC | TTGGCCGCGA | TGTTTAGCTG |
| 8581 | CACGTATTCC | CGCGCAACGC | ACCGCCATTG | GGGAAAGACG | GTGGTCCGCT | CGTGGGGCAC |
| 8641 | CAGGTGCACG | CGCCAACCGC | GTTGTGTCAG | GGTGACAAGG | TCAACGCTGG | TGGCTACCTC |

Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|-------|------------|-------------|------------|------------|------------|-------------|
| 8701 | TCCGCGTAGG | CGCTCGTTGG | TCCAGCAGAG | GCGGCGGCCC | TTGCGCGAAC | AGAATGGGGG |
| 8761 | TAGTGGGTCT | AGCTGGGTCT | CGTCCGGGGG | GTCTGGGTCC | ACGGTAAAGA | CCCCGGGCGAG |
| 8821 | CAGGCGGGCG | TCGAAGTAGT | CTATCTTGCA | TCCTTGCAAG | TCTAGGGCCT | GCTGCCATGC |
| 8881 | GCGGGCGGCA | AGCGGGGGCT | CGTATGGGTT | GAGTGGGGGA | CCCCATGGCA | TGGGTGGGT |
| 8941 | GAGCGGGGAG | GGGTACATGC | CGCAAATGTC | GTAAACGTAG | AGGGGCTCTC | TGAGTATTCC |
| 9001 | AAGATATGTA | GGGTAGCATC | TTCCACGGCG | GATGCTGGCG | CGCACGTAAT | CGTATAGTTC |
| 9061 | GTGCGAGGGA | GCGAGGAGGT | CGGACCGAG | GTTGCTACGG | GCGGGCTGCT | CTGCTCGGAA |
| 9121 | GACTATCTGC | CTGAAGATGG | CATGTGAGTT | GGATGATATG | GTTGGACGCT | GGAGACGTT |
| 9181 | GAAGCTGGCG | TCTGTGAGAC | CTACCGCGTC | AOGCACGAAG | GAGGCGTAGG | AGTCGGCGAG |
| 9241 | CTTGTGACC | AGCTCGGGCG | TGACCTGCAC | GTCTAGGGCG | CAGTAGTCCA | GGGTTTCCTT |
| 9301 | GATGATGTCA | TACTTATCCT | GTCCCTTTT | TTTCCACAGC | TGCGGGTTGA | GGACAACTC |
| 9361 | TTCCGGGTCT | TTCCAGTACT | CTTGGATCGG | AAACCCGTCG | GCCTCGGAAC | GGTAAGAGCC |
| 9421 | TAGCATGTAG | AACTGGTTGA | CGGCTGGTA | GGCGCAGCAT | CCCTTTTCTA | CGGCTAGCGC |
| 9481 | GTATGCCTGC | GCGGCTTCC | GGAGCGAGGT | GTGGGTGAGC | GCAAAGGTGT | CCCTAACCAT |
| 9541 | GACTTTGAGG | TACTGGTATT | TGAAGTCAGT | GTCGTGCGAT | CGCCCTGCT | CCGAGAGCAA |
| 9601 | AAAGTCGGTG | CGCTTTTGG | AACGGGGTT | TGGCAGGGCG | AAGGTGACAT | CGGTGAAAAG |
| 9661 | TATCTTTCC | GCGCGAGGCA | TAAAGTTCCG | TGTGATGCG | AAGGGTCCCG | GCACCTCGGA |
| 9721 | ACGGTTGTTA | ATTACCTGGG | CGGCGAGCAC | GATCTGCTCG | AAGCCGTTGA | TGTTGTGGCC |
| 9781 | CACGATGTAA | AGTTCCAAGA | AGCGCGGGGT | GCCCTTGATG | GAGGGCAATT | TTTTAAGTTC |
| 9841 | CTGTAGGTG | AGCTCCTCAG | GGGAGCTGAG | CCCGTGTCT | GACAGGGCCC | AGTCTGCAAG |
| 9901 | ATGAGGGTTG | GAAGCGAOGA | ATGAGCTCCA | CAGGTACGG | GCCATTAGCA | TTTGCAGGTG |
| 9961 | GTCCGGAAG | GTCCCTAACT | GGCGACCTAT | GGCCATTTT | TCTGGGGTGA | TGCAGTAGAA |
| 10021 | GGTAAGCGGG | TCTTGTTCOC | AGCGGTCCCA | TCCAAGGTCC | ACGGCTAGGT | CTCGCGGGC |
| 10081 | GGTCACCAGA | GGCTCATCTC | CGCCGAACTT | CATAACCAGC | ATGAAGGGCA | CGAGCTGCTT |
| 10141 | CCCAAAGGCC | CCCATCCAAG | TATAGGTCTC | TACATCGTAG | GTGACAAAGA | GACGCTCGGT |
| 10201 | GCGAGGATGC | GAGCCGATCG | GGARGAAGTG | GATCTCCCGC | CACCAGTTGG | AGGAGTGGCT |
| 10261 | GTTGATGTGG | TGAAAGTAGA | AGTCCCTGCG | ACGGGCGGAA | CACTCGTGCT | GGCTTTTGTA |
| 10321 | AAAACGTGCG | CAGTACTGGC | AGCGGTGCAC | GGCGTGTACA | TCCTGCACGA | GGTTGACCTG |
| 10381 | ACGACCGCGC | ACAAGGAAGC | AGAGTGGGAA | TTTGAGCCCC | TGCGCTGGCG | GGTTTGGCTG |
| 10441 | GTGGTCTTCT | ACTTCGGCTG | CTTGTCTCTG | ACCGTCTGOC | TGCTCGAGGG | GAGTTATGGT |
| 10501 | GGATCGGACC | ACCACGCCGC | GCGAGCCCAA | AGTCCAGATG | TCCGCGGGCG | GCGGTGCGAG |
| 10561 | CTTGATGACA | ACATCGCGCA | GATGGGAGCT | GTCCATGGTC | TGGAGCTCC | GCGGCGACAG |
| 10621 | GTCAGGCGGG | AGCTCCTGCA | GGTTTACCTC | GCATAGCCCG | GTGAGGGCG | GGGCTAGGTC |
| 10681 | CAGGTGATAC | CTGATTTCCA | GCGGCTGGTT | GGTGGCGGGC | TCGATGACTT | GCAAGAGGCC |
| 10741 | GCATCCCCGC | GGCGCGACTA | CGGTACCGCG | CGGCGGGCGG | TGGGCGCGCG | GGGTGTCCTT |
| 10801 | GGATGATGCA | TCTAAAAGCG | GTGACGCGCG | CGGGCCCCCG | GAGGTAGGGG | GGGCTCGGGA |
| 10861 | CCCGCCGGGA | GAGGGGGGCG | GGGCACGTCG | GCGCGCGCGG | CGGGCAGGAG | CTGGTCTGCT |
| 10921 | GCGCGGAGGT | TGCTGGCGAA | CGCGACGACG | CGGCGGTTGA | TCTCTGAAT | CTGGCGCCTC |
| 10981 | TGCGTGAAGA | CGACGGGCCC | GGTGAGCTTG | AACCTGAAAG | AGAGTTGAC | AGAATCAATT |
| 11041 | TGGGTGTCGT | TGACGGCGGC | CTGGCGCAAA | ATCTCCTGCA | CGTCTCTGA | GTGTCTTTGA |
| 11101 | TAGGCGATTT | CGGCCATGAA | CTGCTCGATC | TCTTCTCTCT | GGAGATCTCC | GCGTCCGGCT |
| 11161 | CGCTCCACGG | TGGCGGCGAG | GTCGTTGGAG | ATGCGGGCCA | TGAGCTGCGA | GAAGGCGTTG |
| 11221 | AGGCCTCCCT | CGTTCCAGAC | GCGGCTGTAG | ACCACGCCCC | CTTCGGCATC | GCGGCGCGCG |
| 11281 | ATGACCACCT | GCGCGAGATT | GAGCTCCACG | TGCGGGCGGA | AGACGGCGTA | GTTTCGCAGG |
| 11341 | CGCTGAAAGA | GGTAGTTGAG | GGTGGTGGCG | GTGTGTTCTG | CCACGAAGAA | GTACATAACC |
| 11401 | CAGCGTCGCA | ACGTGGATTTC | GTTGATATCC | CCCAAGGCCT | CAAGGCGCTC | CATGGCCTCG |
| 11461 | TAGAAGTCCA | CGGCGAAGTT | GAAAACTGG | GAGTTGCGCG | CCGACACGGT | TAACCTCTCC |
| 11521 | TCCAGAAGAC | GGATGAGCTC | GGCGACAGTG | TGCGGCACCT | CGCGCTCAA | GGCTACAGGG |
| 11581 | GCCTCTTCTT | CTTCAATCTC | CTCTTCCATA | AGGGCCTCC | CTTCTTCTTC | TTCTTCTGGC |
| 11641 | GCGGTTGGGG | GAGGGGGGAC | ACGGCGGGCA | CGACGGCGCA | CCGGAGGGCG | GTCGACAAAG |
| 11701 | CGCTCGATCA | TCTCCCGCG | GCGACGGCGC | ATGCTCTCGG | TGACGGCGCG | GCCGTTCTCG |
| 11761 | GCGGGGCGCA | GTTGGAAGAC | GCCGCCCCGC | ATGTCCCGGT | TATGGGTTGG | CGGGGGGCTG |
| 11821 | CCGTGCGGCA | GGGATACGGC | GCTAACGATG | CATCTCAACA | ATTGTTGTCT | AGGTACTCCG |
| 11881 | CCACCGAGGG | ACCTGAGCGA | GTCCGATCG | ACCGGATCGG | AAAACCTCTC | GAGAAAGGGG |
| 11941 | TCTAACCAGT | CACAGTCGCA | AGGTAGGCTG | ACCACGCTGG | CGGCGGCGAG | CGGCTGGGGG |
| 12001 | TGCGGGTTGT | TTCTGGCGGA | GGTGCTGCTG | ATGATGTAAT | TAAAGTAGGC | GCTCTTGAGA |
| 12061 | CGCCGGATGG | TGACAGAAAG | CACCATGTCC | TTGGGTCCCG | CCTGCTGAAT | GCGCAGGCGG |

Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|-------|------------|-------------|------------|------------|------------|------------|
| 12121 | TGGGCCATGC | CCCAGGCTTC | GTTTTGACAT | CGGCGCAGGT | CTTTGTAGTA | GTCTTGCATG |
| 12181 | AGCCTTTCTA | CCGGCACTTC | TTCTTCTCCT | TCCTCTTGTC | CTGCATCTCT | TGCATCTATC |
| 12241 | GCTACGGCGG | CGGCGGAGTT | TGGCGGTAGG | TGGCGCCCTC | TTCTTCCCAT | GCGTGTGACC |
| 12301 | CCGAAGCCCC | TCATCGGCTG | AAGCAGGGCC | AGGTCCGGGA | CAACGCGCTC | GCCTAATATG |
| 12361 | GCCTGCTGCA | CCTGCGTGAG | GSTAGACTGG | AAGTCATCCA | TGTCCACAAA | GCGGTGGTAT |
| 12421 | GCGCCCGTGT | TGATGGTGTA | AGTGCACTTG | GCCATAAOCG | ACCAGTTAAC | GGTCTGGTGA |
| 12481 | CCCGGCTOCG | AGAGCTCGGT | GTACCTGACA | CGCGAGTAAG | CCCTTGAGTC | AAAGACGTAG |
| 12541 | TGGTTGCAAG | TCCGCACCCAG | GTACTGATAT | CCCACCAAAA | AGTGCGGCGG | CGGCTGGGCG |
| 12601 | TAGAGGGGCC | AGCGTAGGGT | GGCCGGGGCT | CGCGGGGGGA | GGTCTTCCAA | CATAAGGCGA |
| 12661 | TGATATCCGT | AGATGTACCT | GGACATCCAG | GTGATGCCGG | CGGCGGTGGT | GGAGCGCGCC |
| 12721 | GGAAAGTCGC | GGACGCGGTT | CCAGATGTTG | CGCAGCGGCA | AAAAGTGCTC | CATGGTCCGG |
| 12781 | ACGCTCTGGC | CGGTGAGGCG | TGCGCAGTGG | TTGACGCTCT | AGACCGTGCA | AAAGGAGAGC |
| 12841 | CTGTAAGCGG | GCACTCTTCC | GTGGTCTGGT | GGATAAATTC | GCAAGGGTAT | CATGGCGGAC |
| 12901 | GACCGGGGTT | CGAACCCCGG | ATCCGGCCGT | CGCCGCTGAT | CCATGGGGTT | ACCGCCCGCG |
| 12961 | TGTCGAACCC | AGGTGTGCGA | CGTCAGACAA | CGGGGAGAGG | CTCCTTTTGG | CTTCCCTCCA |
| 13021 | GGCGGGGCGG | CTGCTGCGCT | AGCTTTTPTG | GCCACTGGCC | GCGCGCGGCG | TAAGCGGTTA |
| 13081 | GGCTGGAAAG | CGAAAGCATT | AAGTGCGCTC | CTCCCTGTAG | CCGAGGGGTT | ATTTTCCAAG |
| 13141 | GGTTAGTGGC | CAGGACCCCG | GGTTGAGTGC | TGGGGCGGGC | CGGACTGCGG | CGAAGCGGCG |
| 13201 | TTTGCCCTCC | CGTCATGCAA | GACCCCGCTT | GCAAATTCCT | CGGAAACAG | GGACGAGCCC |
| 13261 | CTTTTGTGCT | TTTCCAGAT | GCATCCGGTG | CTGCGGCAGA | TGCGCCCCCG | TCCTCAGCAG |
| 13321 | CGGCAAGAGC | AAGAGCAGCG | GCAGACATGC | AGGGCACCCT | CCCCCTTCTC | TACCGCGTCA |
| 13381 | GGAGGGGCAA | CATCCGCGGC | TGACCGCGCG | GCAGATGGTG | ATTACGAACC | CCCGCGGCGC |
| 13441 | CGGGCCCGGC | ACTACCTGGA | CTTGGAGGAG | GGCGAGGGCC | TGGCGCGGCT | AGGAGCGCCC |
| 13501 | TCTCTGAGCG | GACACCCAAG | GGTGACGCTG | AAGCGTGACA | CGCGGAGGCG | GTACGTGCGG |
| 13561 | CGGCAGAAC | TGTTTTCGGA | CGCGAGGGGA | GAGGAGCCCC | AGGAGATGCG | GGATCGAAAG |
| 13621 | TTCCACGCAG | GGCGCGAGTT | GCGGCATGGC | CTGAACCGCG | AGCGGTTGCT | GCGCGAGGAG |
| 13681 | GACTTTGAGC | CCGACGCGCG | GACCCGGATT | AGTCCCGGCG | GCGCACACGT | GCGGACCGCC |
| 13741 | GACCTGGTAA | CCGCGTACGA | GCAGACGGTG | AACCAGGAGA | TTAACTTTCA | AAAAAGCTTT |
| 13801 | AACAACCACG | TGCGCACGCT | TGTGGCGCGC | GAGGAGGTGG | CTATAGGACT | GATGCACTCT |
| 13861 | TGGGACTTTG | TAAGCGCGCT | GGAGCAAAAC | CCAAATAGCA | AGCCGCTCAT | GGCGCAGCTG |
| 13921 | TTCCCTTATG | TGCAGCACAG | CAGGACAAAC | GAGGCATTCA | GGGATGCGCT | GCTAAACATA |
| 13981 | GTAGAGCCCG | AGGGCGGCTG | GCTGCTCGAT | TTGATAAACA | TTCTGCAGAG | CATAGTGGTG |
| 14041 | CAGGAGCGCA | GCTTCAGCCT | GGCTGACAAG | GTGGCCGCCA | TTAACTATTG | CATGCTCACT |
| 14101 | CTGGGCAAGT | TTTACGCCCC | CAAGATATAC | CATACCCCTT | ACGTTCCCAT | AGACAAGGAG |
| 14161 | GTAAAGATCG | AGGGGTTCTA | CATGGCTATG | GCGTTGAAGG | TGCTTACCTT | GAGCGACGAC |
| 14221 | CTGGGCGTTT | ATCGCAACGA | GCGCATCCAC | AAGGCCGTGA | GCGTGAGCCG | GCGGCGCGAG |
| 14281 | CTCAGCGACC | GCGAGCTGAT | GCACAGCCTG | CAAAGGGCCC | TGGCTGGCAC | GGGCAGCGGC |
| 14341 | GATAGAGAGG | COGAGTCCTA | CTTTGACGCG | GGCGCTGACC | TGCGCTGGGC | CCCAAGCCGA |
| 14401 | CGCGCCCTGG | AGGCAGCTGG | GGCCGGACCT | GGGCTGGGCG | TGGCACCCCG | GCGCGCTGGC |
| 14461 | AACGTCCGCG | GCGTGAGGGA | ATATCAOGAG | GACGATGAGT | ACGAGCCAGA | GGACGGCGAG |
| 14521 | TACTAAGCGG | TGATGTTTCT | GATCAGATGA | TGCAAGACGC | AACGGACCCG | GCGGTGCGGG |
| 14581 | CGGCGCTGCA | GAGCCAGCCG | TCCGGCCTTA | ACTCCACGGA | CGACTGGCGC | CAGGTCAATG |
| 14641 | ACCGCATCAT | GTCGCTGACT | GCGCGTAACC | CTGACGCGTT | CCGGCAGCAG | CCGCAGGCCA |
| 14701 | ACCGGCTCTC | CGCAATTCTG | GAAGCGGTGG | TCCCGGCGCG | CGCAAACCCC | ACGCACGAGA |
| 14761 | AGGTGCTGGC | GATCGTAAAC | GCGCTGGCGG | AAAACAGGGC | CATCCGGCCC | GATGAGGCGG |
| 14821 | GCCTGGTCTA | CGACGCGCTG | CTTCAGCGCG | TGGCTCGTTA | CAACAGCGGC | AACGTGCAGA |
| 14881 | CCAACCTGGA | CCGGCTGGTG | GGGGATGTGC | GCGAGGCCGT | GGCGCAGCGT | GAGCGCGCGC |
| 14941 | AGCAGCAGGG | CAACCTGGGC | TCCATGGTTG | CACTAAACGC | CTTCTGAGT | ACACAGCCCG |
| 15001 | CCAACGTGCC | GCGGGGACAG | GAGGACTACA | CCAACCTTGT | GAGCGCACTG | CGGCTAATGG |
| 15061 | TGACTGAGAC | ACCGCAAAGT | GAGGTGTACC | AGTCCGGGCC | AGACTATTTT | TTCCAGACCA |
| 15121 | GTAGACAAGG | CCTGCAGACC | GTAAGTCTGA | GCCAGGCTTT | CAAGAACTTG | CAGGGGCTGT |
| 15181 | GGGGGGTGCG | GGCTCCCAAC | GGCGACCGCG | CGACCGTGTC | TAGCTTGCTG | ACGCCCAACT |
| 15241 | CGCGCCTGTT | GCTGCTGCTA | ATAGOGCCCT | TCACGGACAG | TGGCAGCGTG | TCCCGGGACA |
| 15301 | CATACCTAGG | TCACTGTCTG | ACACTGTACC | GCGAGGCCAT | AGGTCAGGCG | CATGTGGACG |
| 15361 | AGCATACTTT | CCAGGAGATT | ACAAGTGTC | GCGCGCGGCT | GGCGCAGGAG | GACACGGGCA |
| 15421 | GCCTGGAGGC | AACCTGGAAC | TACCTGTGTA | CCAACCGGCG | GCAGAAGATC | CCCTCGTTGC |
| 15481 | ACAGTTTAAA | CAGCGAGGAG | GAGCGCATCT | TGCGCTATGT | GCAGCAGAGC | GTGAGCCTTA |

Nucleotide Sequence Analysis (cont.)

15541 ACCTGATGCG CGACGGGGTA ACGCCAGCG TGGCGCTGGA CATGACCGCG OGCAACATGG
15601 AACCGGGCAT GTATGCCCTCA AACCGGCCGT TTATCAATCG CCTAATGGAC TACTTGCAATC
15661 GCGCGGGCCG CGTGAAACCCC GAGTATTTCA CCAATGCCAT CTTGAACCOG CACTGGCTAC
15721 CGCCCCCTGG TTCTTACACC GGGGGATTTC AGGTGCCCGA GGGTAACGAT GATTTCTCT
15781 GGGACGACAT AGACGACAGC GTGTTTTCCC OGCAACCGCA GACCCTGCTA GATTGCAAC
15841 AGCGGAGCA GGCAGAGGCG GCGCTGCGAA AGGAAAGCTT CCGCAGGCCA AGCAGCTTGT
15901 CCGATCTAGG CGCTGCGGCC CGCGGTCAG ATCGGAGTAG CCCATTTCOA AGCTTGATAG
15961 GGTCTTTTAC CAGCACTCGC ACCACCCGCC CGCGCTGCT GGGCGAGGAG GAGTACCTAA
16021 ACAACTCGCT GCTGCAGCCG CAGCGCGAAA AGAACCTGCC TCGGCATTTC CCCAACAAOG
16081 GGATAGAGAG CCTAGTGGAC AAGATGAGTA GATGGAAGAC GTATGCGCAG GAGCACAGGG
16141 ATGTGCCCGG CCCGCGCCCG CCCACCCGTC GCAGACGACA GCAGCGTCCT GGATTTGGGA GGGAGTGGCA
16201 TGTGGGAGGA CGATGACTCG GCAGACGACA CCCAGGCTGG GGAGAATGTT TTAACAAAAA AAAAAAAG
16261 ACCCGTTTGC GCACCTTCGC CCCAGGCTGG CATGGCACCG AGCGTTGGTT TTCTTGATT
16321 CATGATGCAA AATAAAAAAC TCACCAAGGC GAGGAAGGTC CTCCTCCCTC CTACGAGAGC
16381 CCCCTTAGTA TGCAGCGCGC GCGGATGTAT GAGGAAGGTC CTCCTCCCTC CTACGAGAGC
16441 GTGGTGAGCG CGGCGCCAGT GCGCGCGCGG CCGGCTGCTG CCGGCTGCTG GAGAGAACAG CATCOGTTAC
16501 CCGCGGTTTG TGCCCTCCGG GTACCTCGCG CGACACCACC CGTGTGTACC TTGTGGACAA CAAGTCAACG
16561 TCTGAGTTGG CACCCCTATT CCAGAAOGAC CACAGCAACT TTCTAACAC GGTCAATCAA
16621 GATGTGGCAT ACAGCCCGGG GCGACCTGAA AACCATCCTG CATACCAACA TCAATCTTGA CGACGTTGG
16681 AACAACTGACT ACAGCCCGGG GCGACCTGAA AACCATCCTG CATACCAACA TCAATCTTGA CGACGTTGG
16741 CACTGGGGCG GCGACCTGAA AACCATCCTG CATACCAACA TCAATCTTGA CGACGTTGG
16801 ATGTTTACCA ATAAGTTTAA GCGCGGGGTG ATGCTGTGCG GCTCGCTTAC TAAGGACAAA
16861 CAGGTGGAGC TGAAATATGA GTGGGTGGAG TTCACGCTGC CCGAGGGCAA CTACTCGAG
16921 ACCATGACCA TAGACCTTAT GAACAACGCG ATCGTGGAGC ACTACTTGAA AGTGGGCGAG
16981 CAGAACGGGG TTCTGGAAAG CGACATCGGG GTAAAGTTTG ACACCCGCAA CTTGAGACTG
17041 GGGTTTGACC CAGTCACTGG TCCTGTGATG CCTGGGGTAT ATACAAACGA AGCCTTCCAT
17101 CCAGACATCA TTTTGCTGCC AGGATGCGGG GTGGACTTCA CCCACAGCCG CCGTACGAA
17161 TTGTTGGGCA TCCGCAAGOG GCAACCCCTT CAGGAGGGCT TTAGGATCAC CTACCATGAC
17221 CTGGAGGGTG GTAACATTCC CGCACTGTTG GATGTGGACG CCTACCAGGC AAGCTTAAAA
17281 GATGACACOG AACAGGGGCG GGATGGGCGA GCGCGCGCGA ACAACAGTGG CAGCGGCGCG
17341 GAAGAGAACT CCAACGCGGC AGCGCGCGCA ATGCGACCGG TGGAGGACAT GAACGATCAT
17401 GCCATTTCGG GCGACACCTT TGCCACACGG GCGGAGGAGA AGCGCGCTGA GGCCGAGGCA
17461 GCGGCAGAAG CTGCGCGCCC CGCTGCGCAA AAACGCAGTT ACAACCTAAT AAGCAATGAC
17521 GTGATCAAA CCGTGACAGA GGACAGCAAG CTTGCATACA ACTACGGCGA CCCTCAGACC
17581 AGCACCTTCA CCCAGTACCG CAGCTGGTAC CTTGCATACA ACTACGGCGA CCCTCAGACC
17641 GGGATCCGCT CATGGACCCCT CTTTGCATC CTTGACGTAA CCTGCGGCTC GGAGCAGGTC
17701 TACTGGTCTG TGCCAGACAT GATGCAAGAC CCCGTGACCT TCCGCTCCAC GAGCCAGATC
17761 AGCAACTTTC CCGTGGTGGG CGCCGAGCTG TTGCCCGTGC ACTCCAAGAG CTTCTACAAC
17821 GACCAGGCGG TCTACTCCCA GCTCATCCCG CAGTTTACCT CTCTGACCCA CGTGTTCAT
17881 CGCTTTCCCG AGAACCCAGT TTTGGGCGCG CCGCCAGCCC CCACCATCAC CACCGTCAGT
17941 GAAAACGTTT CTGCTCTCAC AGATCACGGG ACGCTACCGC TGCGCAACAG CATCGGAGGA
18001 GTCCAGCGAG TGACCATTAC TGACGCCAGA CGCCGCACCT GCGCCCTACG TTACAAGGCC
18061 CTGGGCATAG TCTCGCCGCG CGTCCATATC AGCCGCACTT TTTGAGCAAA CATGTCCATC
18121 CTTATATGCG CCAGCAATAA CACAGGCTGG GGCCTGCGCT TCCCAAGCAA GATGTTTGGC
18181 GGGGCAAGA AGCGCTCCGA CCAACACCCA GTGCGCGTGC GCGGGCACTA CCGCGCGCCC
18241 TGGGGCGGCG ACAACCGGG CCGCACTGGG CGCACACCG TCGATGACGC CATTGACGCG
18301 GTGGTGGAGG AGGCGCGCAA CTACACGCCC ACGCCGCCAC CAGTGTCAC AGTGGACGCG
18361 GCCATTGAGA CCGTGGTGGC CGGAGCCCGG CGTTATGCTA AAATGAAGAG ACGCGGAGG
18421 CGCGTAGCAC GTCGCCACCG CCGCCGACCC GGCACCTGCC CCCAACGCGC GCGCGCGGCC
18481 CTGCTTAACC GCGCACGTCG CACCGGCGGA CGGGCGGCCA TCGCGGCGCG TCGAAGGCTG
18541 GCGCGGGTA TTGTCACTGT GCGCCCCAGG TCCAGGCGAC GAGCGGCGCG CGCAGCAGCC
18601 GCGGCCATTA GTGCTATGAC TCAGGGTGGC AGGGGCAACG TGTACTGGGT GCGCGACTCG
18661 GTTAGCGGCC TGCGCGTGCC CGTGCGCAC CCGCCCCCGC GCAACTAGAT TGCAAGAAAA
18721 AACTACTTAG ACTCGTACTG TTGTATGTAT CCAGCGCGCG CGGCGCGCAA CGAAGCTATG
18781 TCCAAGCGCA AAATCAAAGA AGAGATGCTC CAGGTATCG CGCGGAGAT CTATGCGCCC
18841 CCGAAGAAGG AAGAGCAGGA TTACAAGCCC CGAAAGCTAA AGCGGGTCAA AAAGAAAAAG
18901 AAAGATGATG ATGATGATGA ACTTGACGAG GAGGTGGAAC TGCTGCACGC AACCAGCGCC

Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|-------|-------------|------------|-------------|------------|-------------|-------------|
| 18961 | AGGCGGCGGG | TACAGTGGAA | AGGTGACGCC | GTAAGACGTG | TTTTGGGACC | GGGCAACCACC |
| 19021 | GTAAGTTTTA | CGCCCCGTGA | GCGCTCCACC | CGCACCTACA | AGCGCGTGTA | TGATGAGGTG |
| 19081 | TACGGCGAOG | AGGACCTGCT | TGAGCAGGCC | AACGAGCGCC | TCGGGAGGTT | TGCCTAOGGA |
| 19141 | AAGCGGCATA | AGGACATGTT | GGGGTTGGCG | CTGGACGAGG | GCAACCCAAC | ACCTAGCCTA |
| 19201 | AAGCCCGTGA | CACTGCAGCA | GGTGTGCCCC | ACGCTTGAC | CGTCCGAAGA | AAAGCGGGCC |
| 19261 | CTAAAGCGCG | AGTCTGGTGA | CTTGGCACCC | ACCGTGCAGC | TGATGGTACC | CAAGCGCCAG |
| 19321 | CGACTGGAAG | ATGTCTTGG | AAAAATGACC | GTGGAGCCTG | GGCTGGAGCC | CGAGGTCCGC |
| 19381 | GTGCGGCCAA | TCAAGCAGGT | GGCACCCGGA | CTGGGCGTGC | AGACCGTGG | CGTTCAGATA |
| 19441 | CCCACCACCA | GTAGCACTAG | TATTGGCACT | GCCACAGAGG | GCATGGAGAC | ACAAACGTCC |
| 19501 | CCGGTTGCCCT | CGGCGGTGGC | AGATGCCGCG | GTGCAGGCGG | CGGCTGGCGC | CGCGTCCAAA |
| 19561 | ACCTCTAOCG | AGGTGCAAA | GGACCCGTGG | ATGTTTCGGG | TTTCAGCCCC | CCGGCGCCCCG |
| 19621 | CGCGTTTCCA | GGAAGTACGG | CACCGCCAGC | GCACTACTGC | CCGAATATGC | CCTACATCCT |
| 19681 | TCCATCGCGC | CTACCCCCGG | CTATCGTGGC | TACACCTACC | GCCCCAGAAG | ACGAGCGACT |
| 19741 | ACCCGACGCC | GAACCACCAC | TGGAACCCGC | CGCGCGCGTC | GCGGTGCGCA | GCCCCGTGCTG |
| 19801 | GCCCCGATTT | CGGTGCGCAG | GGTGGCTCGC | GAAGGAGGCA | GGACCCCTGGT | GCTGCCAACA |
| 19861 | GCGCGCTACC | ACCCGAGCAT | CGTTTAAAG | CGGGTCTTTG | TGGTTCTTTC | AGATATGGCC |
| 19921 | CTCACCTGCC | GCGTCCGTTT | CCCGGTGCGC | GGATTCCGAG | GAAGAATGCA | CCGTAGGAGG |
| 19981 | GGCATGGCCG | GCCACGGCCT | GACGGCGGCG | ATGCGTGTGT | CGCACCAACG | GCGGGGGGCG |
| 20041 | GCGTGGCACC | GTCCGATGCG | CGGCGGTATC | CTGCCCCCTC | TTATTCCACT | GATCGCCGCG |
| 20101 | GCGATTGGCG | CCGTGCCCCG | AATTGCATCC | GTGGCCTTGC | AGGCGCAGAG | ACACTGATTA |
| 20161 | AAAACAAGTT | GCATGTGGAA | AAATCAAAAT | AAAAAGTCTG | GAGTCTCAGC | CTCGCTTGCT |
| 20221 | CCTGTAACTA | TTTTGTAGAA | TGGAAGACAT | CAACTTTGCG | TCTCTGGCCC | CGCGACACCG |
| 20281 | CTCGCGCCCG | TTCATGGGAA | ACTGGCAAGA | TATCGGCACC | AGCAATATGA | GCGGTGGGCG |
| 20341 | CTTCAGCTGG | GGCTCGCTGT | GGACGGGCAT | TAAAAATTTT | GGTTCCACCA | TTAAGAACTA |
| 20401 | TGGCAGCAAG | GCCTGGAACA | GCAGCACAGG | CCAGATGCTG | AGGGACAAGT | TGAAAGAGCA |
| 20461 | AAATTTCCAA | CAAAAGGTGG | TAGATGGCCT | GGCCTCTGGC | ATTAGCGGGG | TGTTGGACCT |
| 20521 | GGCCAACCAG | GCAGTGCAAA | ATAAGATTAA | CAGTAAGCTT | GATCCCCGCG | CTCCCGTAGA |
| 20581 | GGAGCCTCCA | CCGGCCGTGG | AGACAGTGTG | TCCAGAGGGG | CGTGGCGAAA | AGCGTCCGCG |
| 20641 | CCCCGACAGG | GAAGAAACTC | TGCTGAAGCA | AATAGATGAG | CCTCCCTCGT | ACGAGGAGGC |
| 20701 | ACTAAAGCAA | GGCCTGCCCA | CCACCCGTCC | CATCGGCCCC | ATGGCTACCG | GAGTGTGGG |
| 20761 | CCAGCACACA | CCTGTAAOCG | TGGACCTGCC | TCCCCCGGCT | GACACCCAGC | AGAAACCTGT |
| 20821 | GCTGOCAGGG | CCGTCCGCGG | TGTGTGTAAC | CCGCCCCTAG | CGCGGCTCCC | TGCGCCGTGC |
| 20881 | CGCCAGCGGT | CCGCGATCGA | TGCGGCCCGT | AGCCAGTGGC | AACTGGCAAA | GCACACTGAA |
| 20941 | CAGCATCGTG | GGTCTGGGGG | TGCAATCCCT | GAAGCGCCGA | CGATGCTTCT | AAATAGCTAA |
| 21001 | CGTGTGCTAT | GTGTATGTA | TGCGTCCATG | TCGCCCCCAG | AGGAGCTGCT | GAGCCGCGCT |
| 21061 | CGGCCCCGCTT | TCCAAGATGG | CTACCCCTTC | GATGATGCGG | CAGTGGTCTT | ACATGCACAT |
| 21121 | CTCGGGCCAG | GACGCCTCGG | AGTACCTGAG | CCCCGGGCTG | GTGCAGTTTG | CCCCGGCCAC |
| 21181 | CGAGACGTAC | TTCAGCCTGA | ATAACAAGTT | TAGAAACCCC | ACGGTGGCAC | CTACGCACGA |
| 21241 | CGTAACCACA | GACCGGTCCC | AGCGTTTGAC | GCTGCGGTTT | ATCCCTGTGG | ACCGCGAGGA |
| 21301 | TACCGCGTAC | TCGTACAAAG | CGCGGTTTAC | CCTGGCTGTG | GGTGACAACC | GTGTGCTTGA |
| 21361 | TATGGCTTCC | ACGTACTTTG | ACATCCGCGG | CGTGTCTGG | AGGGGGCCTA | CTTTTAAGCC |
| 21421 | CTACTCOGGC | ACTGCCTACA | ACGCTCTAGC | TCCCAAGGGC | GCTCCTAACT | CCTGTGAGTG |
| 21481 | GGAACAAACC | GAAGATAGCG | GCCGGGCGAGT | TGCCGAGGAT | GAAGAAGAGG | AAGATGAAGA |
| 21541 | TGAAGAAGAG | GAAGAAGAAG | AGCAAAAAGC | TCGAGATCAG | GCTACTAAGA | AAACACATGT |
| 21601 | CTATGCCCCAG | GCTCCTTTGT | CTGGAGAAAC | AATTACAAAA | AGCGGGCTAC | AAATAGGATC |
| 21661 | AGACAATGCA | GAAACACAAG | CTAAACCTGT | ATAOGCAGAT | CCTTCCTATC | AACCAGAACC |
| 21721 | TCAAAATTGGC | GAATCTCAGT | GGAACGAAAC | TGATGCTAAT | GCGGCAGGAG | GGAGAGTGCT |
| 21781 | TAAAAAAACA | ACTCCCATGA | AACCATGCTA | TGGATCTTAT | GCCAGGCCTA | CAAAATCCTTT |
| 21841 | TGGTGGTCAA | TCCGTTCTGG | TTCCGGATGA | AAAAGGGGTG | CCTCTTCCAA | AGGTTGACTT |
| 21901 | GCAATTCTTC | TCAAATACTA | CCTCTTTGAA | CGACCGGCA | GGCAATGCTA | CTAAACCAAA |
| 21961 | AGTGGTPTTG | TACAGTGAAG | ATGTAAATAT | GGAAACCCCA | GACACACATC | TGTCTTACAA |
| 22021 | ACCTGGAAAA | GGTGATGAAA | ATTCTAAAGC | TATGTTGGGT | CAACAATCTA | TGCCAAACAG |
| 22081 | ACCCAATTAC | ATTGCTTTCA | GGGACAATTT | TATTGGCCTA | ATGTATTATA | ACAGCAGTGG |
| 22141 | CAACATGGGT | GTTCTTGCTG | GTCAGGCATC | CGAGCTAAAT | GCCGTGGTAG | ATTTGCAAGA |
| 22201 | CAGAAACACA | GAGCTGTCTT | ATCAACTCTT | GCTTGATTCC | ATAGGTGATA | GAACCAGATA |
| 22261 | TTTTTCTATG | TGGAATCAGG | CTGTAGACAG | CTATGATCCA | GATGTTAGAA | TCATTGAAAA |
| 22321 | CCATGGAACT | GAGGATGAAT | TGCCAAATTA | TGTTTTTCCT | CTTGGGGGTA | TTGGCGTAAC |

Nucleotide Sequence Analysis (cont.)

22381 TGACACCTAT CAAGCTATTA AGGCTAATGG CAATGGCTCA GCGGATAATG GAGATACTAC
22441 ATGGACAAAA GATGAAACTT TTGCAACACG TAATGAAATA GGAGTGGGTA ACAACTTTGC
22501 CATGGAAATT AACCTAAATG CCAACCTATG GAGAAATTTT CTTTACTCCA ATATTGCGCT
22561 GTACCTGCCA GACAAGCTAA AATACAACCC CACCAATGTG GAAATATCTG ACAACCCCAA
22621 CACCTACGAC TACATGAACA AGCGAGTGGT GGCTCCCGGG CTGTAGACT GCTACATTAA
22681 CCTGGGGCG CGCTGGTCTC TGGACTACAT GTTGGGAAAC GCGCGCTAAG TGCCCTTTCA
22741 CAATCGGGC CTCGGTTATC GCTCCATGTT GTTGGGAAAC GCGCGCTAAG TGCCCTTTCA
22801 CATTCAAGTG CCCCAAAAGT TTTTGGCCAT TAAAAACCTC CTCTCTCTGC CAGGCTCATA
22861 TACATATGAA TGGAACTTCA GGAAGGATGT TAACATGGTT CTGCAGAGCT CTCTGGGAAA
22921 CGATCTTAGA GTTGACGGGG CTAGCATTAA GTTTGACAGC ATTTGTCTTT ACGCCACCTT
22981 CTTCCCCATG GCCCACAACA CGGCTTCCAC CGCCAACATG CTATACCCCA TACCGGCCAA
23041 CGACCAGTCC TTAAATGACT ACCTTTTCCG CGCCAACATG CTATACCCCA TACCGGCCAA
23101 CGCCACCAAC GTGCCCATCT CCATCCCATC GCGCAACTGG GCAGCATTTT GCGGTTGGGC
23161 CTTACACGCG TTGAAGACAA AGGAAACCCC TTCCCTGGGA TCAGGCTACG ACCCTTACTA
23221 CACCTACTCT GGCTCCATAC CATACTTGA CGGAACCTTC TATCTTAATC ACACCTTTAA
23281 GAAGGTGGCC ATTACCTTTG ACTCTTCTGT TAGCTGGCGG GGCTACAACG TAGCTCAGTG
23341 TCCCAATGAG TTTGAGATTA AACGCTCAGT TCCTGGTGCA GATGTTGGCC AACTACAATA TTGGCTACCA
23401 CAACATGACC AAGGACTGGT TCCTGGTGCA GATGTTGGCC AACTACAATA TTGGCTACCA
23461 GGGCTTCTAC ATTCCAGAAA GCTACAAGGA CCGCATGTAC TCGTTCTTCA GAACTTTCCA
23521 GCCCATGAGC CGGCAAGTGG TTGACGATAC TAAATACAAG GAGTATCAGC AGGTTGGAAT
23581 TCTTCACCAG CATAACAACCT CAGGATTCTG AGGCTACCTC AAAACOGGGG TTGACAGTAT
23641 ACAGGCTTAC CCCGCCAAGC TGCCCTACCC ACTAATAGGC ATCCCATTTT CCAGTAACCT
23701 TACCCAGAAA AAGTTTCTTT GCGCACTCA CAGACCTGGG CCAAAACCTT CTCTACGCCA ACTCOGCCA
23761 TATGTCCATG GGCGCACTCA CAGACCTGGG CCAAAACCTT CTCTACGCCA ACTCOGCCA
23821 CGCGCTAGAC ATGACTTTTG AGGTGGATCC CATGGAAGAG CCCACCTTTC TTTATGTTTT
23881 GTTTGAAGTC TTTGACGTGG TCCGTGTGCA CCAGCCGCAC CGCGCGTCA TCGAGACCGT
23941 GTACCTGGCG ACGCCCTTCT CGSCCGGCAA CGCCACAACA AAAGCCATTG TCAAAGATCT
24001 AACACAGCT GCGCCATGG CCATATTTTT TGGGCACCTA TGACAAGCGC TTTCCAGGCT TTGTTTCTCC
24061 TGGTTGTGGG CCATATTTTT TAGTCAATAC GGCCGGTGGC GAGACTGGGG GCGTACACTG
24121 ACACAAGCTC GCCTGGGAACC CGCGCTCAAA AACATGCTAC CTCTTTGAGC CTTTGGCTT
24181 GATGGCCTTT GCCTGGGAACC CGCGCTCAAA AACATGCTAC CTCTTTGAGC CTTTGGCTT
24241 TTCTGACCAA CGACTCAAGC AGGTTTACCA GTTTGAGTAC GAGTCACTCC TGGCCGCTAG
24301 CGCCATTGCT TCTTCCCCCG ACCGCTGTAT CTGCTGCATG TTTCTCCACG CCTTTGCCAA
24361 GGGGCCCAAC TCGGCGCGCT ATCACAACCC CACCATGAAC CTTATTACCG GGGTACCCAA
24421 CTGGCCCCAA ACTCCCATGG AGGTACAGCC CACCTTGGCT CGCAACCAAG AACAGCTCTA
24481 CTCCATGCTT AACAGTCCCC AGGTACAGCC CACCTTGGCT CGCAACCAAG AACAGCTCTA
24541 CAGCTTCTCT GAGCGCCACT CGCCCTACTT CCGCAGCCAC AGTGGCCAGA TTAGGACCGC
24601 CACTTCTTTT TGTCACCTGA AAAACATGTA AAAATAATGT TACCCCCAC CCTTGCCGTC
24661 AAGGCAAAATG TTTTATTTG TACACTCTCG GGTGATTATT TACCCCCAC CCTTGCCGTC
24721 TGCGCGGTTT AAAAATCAAA GGGGTTCTGC CGCGCATCGC TATCGCCAC TGGCAGGGAC
24781 ACGTTGCGAT ACTGGTGTG AGTGCTCCAC TTAAGCTCAG GCACAACCAT CCGCGGCAGC
24841 TCGGTGAAGT TTTCACTCCA CAGGCTGCGC ACCATCACCA ACGCGTTTAC CAGGTGCGG
24901 GCCGATATCT TGAAGTCGCA GTTGGGGCCT CCGCCCTGGC GGTGGTGCA CGCTGGCCAG CACGCTCTTG
24961 GGGTTGCAGC ACTGGAACAC TATCAGCGCC CAGGTCTCTC GCGTTGCTCA GGGCGAACGG AGTCAACTTT
25021 TCGGAGATCA GATCCGCGTC TTCCCAAAAA GGGTGCATGC CCAGGCTTTG AGTTGCACTC GCACCGTAGT
25081 GGTAGTTTCC TTCCCAAAAA GGGTGCATGC CCAGGCTTTG AGTTGCACTC GCACCGTAGT
25141 GGCATCAGAA GGTGACCGTG CCCGGTCTGG GCGTTAGGAT ACAGCGCCTG CATGAAAGCC
25201 TTGATCTGCT TAAAAGCCAC CTGAGCCTTT GCGCCTTCAG AGAAGAACAT GCGCAAGAC
25261 TTGCCGGAAG ACTGATTGGC CGGACAGGCC GCGTCATGCA CGCAGCACCT TGGTGGGTG
25321 TTGGAGATCT GCACCACATT TCGGCCCGAC CCGTTCTTCA CGATCTTGGC CTTGCTAGAC
25381 TGCTCCTTCA GCGCGCGCTG CCCGTTTTCG CTCGTACAT CCATTTCAAT CACGTGCTCC
25441 TTATTTATCA TAATGCTCCC GTGTAGACAC TTAAGCTCGC CTTGATCTC AGCGCAGCGG
25501 TGCAGCCACA ACGCGCAGCC CGTGGGCTCG ATCGTCACAA AGGTCTTGT GCTGGTGAAG
25561 TGCAGGTACG CCTGCAGGAA TCGCCCCATC AGCCAGGTCT TGCATACGGC CGCCAGAGCT
25621 GTCAGCTGCA ACCCGCGGTG CTCTCTGTTT GCCTTTAGAT CGTTATCCAC GTGGTACTTG
25681 TCCACTTGGT CAGGCAGTAG CTGGAAGTTT GCCTTTAGAT CGTTATCCAC GTGGTACTTG
25741 TCCATCAACG CGCGCGCAGC CTCCATGCCC TTCTCCACG CAGACACGAT CCGCAGGCTC

Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|-------|------------|------------|------------|-------------|-------------|-------------|
| 25801 | AGCGGGTTTA | TCACCGTGCT | TTCACCTTTC | GCTTCACTGG | ACTCTTCCTT | TTCCTCTTGC |
| 25861 | GTCCGCATAC | CCCGCGCCAC | TGGGTCTGCT | TCATTCAAGC | GCGCACCCTG | GCGCTTACCT |
| 25921 | CCCTTGCCGT | GCTTGATTAG | CACCGGTGGG | TTGCTGAAAC | CCACCATTGT | TAGCGCCACA |
| 25981 | TCTTCTCTTT | CTTCTCTGCT | GTCCAAGATC | ACCTCTGGCG | ATGGCGGGCG | CTCGGCTTGG |
| 26041 | GGAGAGGGGC | GCTTCTTTTT | CTTTTTGAGC | GCAATGGCCA | AATCGCCGT | CGAGGTGAT |
| 26101 | GGCCGCGGGC | TGGTGTGCG | CGGCACCAGC | GCATCTTGTG | ACGAGTCTTC | TTCGTCTCTG |
| 26161 | GACTCGAGAC | GCCGCTCAG | COGCTTTTTT | GGGGGCGCCG | GCGGAGGCGG | CGGCGACGGC |
| 26221 | GACGGGGACG | ACACGTCTTC | CATGCTTGGT | GGACGTGGCG | CGGCACCCTG | TCCGCGCTGG |
| 26281 | GGGGTGGTTT | CGCGCTGCTC | CTCTTCCCGA | CTGGCCATTT | CCTTCTCTTA | TAGGCAGAAA |
| 26341 | AAGATCATGG | AGTCAGTCGA | GAAGGAGGAC | AGCCTAACCG | CCCCCTTTGA | GTTCGCCACC |
| 26401 | ACCGCTCCA | CCGATGCCCG | CAACGCGCCT | ACCACCTTCC | CCGTGAGGGC | ACCCCGCTT |
| 26461 | GAGGAGGAGG | AAGTGATTAT | CGAGCAGGAC | CCAGGTTTGT | TAAGCGAAGA | CGACGAGGAT |
| 26521 | CGCTCAGTAC | CAACAGAGGA | TAAAAAGCAA | GACCAGGACG | ACGCAGAGGC | AAAAGAGGAA |
| 26581 | CAAGTCGGGC | GGGGGGACCA | AAGGCATGGC | GACTACCTAG | ATGTGGGAGA | CGAGCTGCTG |
| 26641 | TTGAAGCATC | TGCAGCGCCA | GTGGGCCATT | ATCTGCGACG | CGTTGCAAGA | GCGCAGCGAT |
| 26701 | GTGCCCCCTG | CCATAGCGGA | TGTCAGCCTT | GCCTAOGAAC | GCCACCTGTT | CTCACCGGCG |
| 26761 | GTACCCCCCA | AAACGCAAGA | AAACGCGACA | TGCGAGCCCA | ACCCGCGCCT | CAACTTCTAC |
| 26821 | CCCGTATTTG | CCGTGCCAGA | GGTGCTTGCC | ACCTATCACA | TCTTTTTTCA | AAACTGCAAG |
| 26881 | ATACCCCTAT | CCTGCGGTGC | CAACCGCAGC | CGAGCGGACA | AGCAGCTGGC | CTTGCGGCAG |
| 26941 | GGCGCTGTCA | TACCTGATAT | CGCCTCGCTC | GACGAAGTGC | CAAAAATCTT | TGAGGGTCTT |
| 27001 | GGACGCGACG | AGAAAACGCG | GGCAAACGCT | CTGCAACAAAG | AAAACAGOGA | AAATGAAAAGT |
| 27061 | CACGTGGGAG | TGCTGGTGGG | ACTTGAGGGT | GACAAACGCG | GCCTAGCGGT | GCTGAAACGC |
| 27121 | AGCATCGAGG | TCACCCACTT | TGCCCTACCG | GCACCTTAAC | TACCCCCCAA | GGTTATGAGC |
| 27181 | ACAGTCATGA | GCGAGCTGAT | CGTGCGCGGT | GCACGACCCC | TGGAGAGGGA | TGCAAACTTG |
| 27241 | CAAGAACAAA | CCGAGGAGGG | CCTACCCGCA | GTGGCGGATG | AGCAGCTGGC | CGCCTGGCTT |
| 27301 | GAGAAGCGCG | AGCCTGCCGA | CTTGGAGGAG | CGAOGCAAGC | TAATGATGGC | CGCAGTGCTT |
| 27361 | GTTACCGTGG | AGCTTGAGTG | CATGACGCGG | TTCTTTGCTG | ACCCGAGAGT | GCAGCGCAAG |
| 27421 | CTAGAGGAAA | CGTTGCACTA | CACCTTTTCG | CAGGGCTAAG | TGCGCCAGGC | CTGCAAAATT |
| 27481 | TCCAACGTGG | AGCTCTGCAA | CTTGCTCTCC | TACCTTGGA | TTTTGACGA | AAACCGCCTC |
| 27541 | GGGCAAAACG | TGCTTCATT | CACGCTCAAG | GGCGAGGCGC | GCGCGGACTA | CGTCCGCGAC |
| 27601 | TGCGTTTACT | TATTTCTGTG | CTACACCTGG | CAAAACGGCA | TGGGCGTGTG | GCAGCAATGC |
| 27661 | CTGGAGGAGC | GCAACCTAAA | GGAGCTGCAG | AAGCTGCTAA | AGCAAAACTT | GAAGGACCTA |
| 27721 | TGGACGGCCT | TCAACGAGCG | CTCCGTGGCC | GCGCACCTGG | CGGACATTAT | CTTCCCGGAA |
| 27781 | CGCCTGCTTA | AAACCTGCA | ACAGGGTCTG | CCAGACTTCA | CCAGTCAAAG | CATGTTGCAA |
| 27841 | AACTTTAGGA | ACTTTATCCT | AGAGCGTTCA | GGAAATCTGC | CCGCCACCTG | CTGTGCGCTT |
| 27901 | CCTAGCGACT | TTGTGCCCAT | TAAGTACCGT | GAATGCCCTC | CGCCGCTTTG | GGGTCACTGC |
| 27961 | TACCTTCTGC | AGCTAGCCAA | CTACCTTCCG | TACCACTCCG | ACATCATOGA | AGACGTGAGC |
| 28021 | GGTGACGGCC | TACTGGAGTG | TCACGTGCGC | TGCAACCTAT | GCACCCCGCA | CCGCTCCCTG |
| 28081 | GTCTGCAATT | CGCAACTGCT | TAGCGAAAGT | CAAATTATOG | GTACCTTTGA | GCTGCGAGGT |
| 28141 | CCCTCGCCTG | ACGAAAAGTC | CGCGGCTCCG | GGGTGAAAC | TCACTCCGGG | GCTGTGAGCG |
| 28201 | TGCGCTTACC | TTGCGAAATT | TGTACCTGAG | GACTACCACG | CCCACGAGAT | TAGGTTCTAC |
| 28261 | GAAGACCAAT | CCCGCCCGCC | AAATGCGGAG | CTTACCGCCT | GCGTCATTAC | CCAGGGCCAC |
| 28321 | ATCCTTGGCC | AATTGCAAGC | CATCAACAAA | GCCCGCCAAG | AGTTTCTGCT | ACGAAAGGGA |
| 28381 | CGGGGGGTTT | ACCTGGACCC | CCAGTCCGCG | GAGGAGCTCA | ACCCAATCCC | CCCGCGCGCG |
| 28441 | CAGCCCTATC | AGCAGCCGCG | GGCCCTTGCT | TCCAGGATG | GCACCCAAAA | AGAAGCTGCA |
| 28501 | GCTGCCGCGG | CCGTCACCCA | CGGACGAGGA | GGAACTACTG | GACAGTCAGG | CAGAGGAGGT |
| 28561 | TTTGACGAG | GAGGAGGAGA | TGATGGAAGA | CTGGGACAGC | CTAGACGAAG | CTTCCGAGGC |
| 28621 | CGAAGAGGTG | TCAGACGAAA | CACCGTCACC | CTGGTTCGCA | TTCCCTTCGC | CGGCGCCCCA |
| 28681 | GAAATTGGCA | ACCGTTCCCA | GCATOGCTAC | AACCTTCGCT | CCTCAGGCGC | CGCCGGCACT |
| 28741 | GCCTGTTCCG | CGACCCAAAC | GTAGATGGGA | CACCACTGGA | ACCAGGGCCG | GTAAGTCTAA |
| 28801 | GCAGCCGCGG | CCGTTAGCCC | AAGAGCAACA | ACAGCGCCAA | GGCTACCGCT | CGTGGCGCGG |
| 28861 | GCACAAGAAC | CCCATAGTTG | CTTGCTTGCA | AGACTGTGGG | GGCAACATCT | CGTCCGCGCG |
| 28921 | CCGCTTTCTT | CTCTACCATC | ACGGCTTGCG | CTTCCCCCGT | AACATCCTGC | ATTACTACCG |
| 28981 | TCATCTCTAC | AGCCCCACT | GCACCGGCGG | CAGCGGCAGC | GGCAGCAACA | GCAGCGGTCA |
| 29041 | CACAGAAGCA | AAGGCGACCG | GATAGCAAGA | CTCTGACAAA | GCCCAAGAAA | TCCACAGCGG |
| 29101 | CGGCAGCAGC | AGGAGGAGGA | GCGCTGCGTC | TGGCGCCCAA | CGAAACCCGTA | TCCACCCCGG |
| 29161 | AGCTTAGAAA | TAGGATTTTT | CCCACCTCTG | ATGCTATATT | TCAACAAAGC | AGGGGCCAAG |

2145641

-93-

Nucleotide Sequence Analysis (cont.)

| | | | | | | |
|-------|-------------|-------------|-------------|------------|-------------|-------------|
| 29221 | AACAAGAGCT | GAAAAATAAAA | AACAGGTCTC | TGCGCTCCCT | CACCCGCAGC | TGCCCTGTATC |
| 29281 | ACAAAAGCGA | AGATCAGCTT | CGGOGCAOCG | TGGAAGACGC | GGAGGCTCTC | TTCAGCAAAT |
| 29341 | ACTGCGCGCT | GACTCTTAAG | GACTAGTTTC | GCGCCCTTTC | TCAAATTTAA | GCGCGAAAAC |
| 29401 | TACGTCACTT | CCAGCGGCCA | CACCGGGGCG | CAGCACCTGT | CGTCAGOGCC | ATTATGAGCA |
| 29461 | AGGAAATTCC | CACGCCCTAC | ATGTGGAGTT | ACCAGCCACA | AATGGGACTT | GCGGCTGGAG |
| 29521 | CTGCCCAAGA | CTACTCAACC | CGAATAAACT | ACATGAGCGC | GGGACCCAC | ATGATATCCC |
| 29581 | GGGTCAAOGG | AATCCGCGCC | CACCGAAAAC | GAATTCCTCT | CGAACAGGCG | GCTATTACCA |
| 29641 | CCACACCTCG | TAATAACCTT | AATCCCGGTA | GTGCGCCGCG | TGCCCTGGTG | TACCAGGAAA |
| 29701 | GTCCCGCTCC | CACCACTGTG | GTACTTCCCA | GAGACGCCCA | GGCCGAAGTT | CAGATGACTA |
| 29761 | ACTCAGGGGC | GCAGCTTGCG | GGCGGCTTTC | GTCACAGGGT | GCGGTGCGCC | GGGCAGGGTA |
| 29821 | TAACTCACCT | GAAAATCAGA | GGGCGAGGTA | TTCAGCTCAA | CGACGAGTCG | GTGAGCTCCT |
| 29881 | CTCTTGGTCT | CGTCCGGGAC | GGGACATTTC | AGATGGGCGG | CGCTGGCGCG | TCCTCATTTA |
| 29941 | CGCCCCGTCA | GGCGATCCTA | ACTCTGCAGA | CCTCGTCTCT | GGAGCGGCGC | TCCGAGGGCA |
| 30001 | TTGGAACTCT | ACAATTTATT | GAGGAGTTTG | TGCGTTGCGT | TTACTTCAAC | CCCTTTTCTG |
| 30061 | GACCTCCCGG | CCACTACCGG | GACCACTTTA | TTCCCAACTT | TGACGGGGTG | AAAGACTCGG |
| 30121 | CGGACGGCTA | CGACTGAATG | ACCAGTGGAG | AGCCAGAGCG | ACTGCGCTCG | ACACACCTCG |
| 30181 | ACCACTGCCG | CCGCCACAAG | TGCTTTGCCG | GCGCTCCCGG | TGAGTTTGTG | TACTTTGAAT |
| 30241 | TGCCCGAAGA | GCATATCGAG | GGCCCCGGCG | ACGGCGTCCG | GCTCACCACC | CAGGTAGAGC |
| 30301 | TTACACGTAG | CCTGATTCCG | GAGTTTACCA | AGCGCCCCCT | GCTAGTGGAG | CGGGAGCGGG |
| 30361 | GTCCCTGTGT | TCTGACCGTG | GTTTGCAACT | GTCTTAACCC | TGGATTACAT | CAAGATCTTT |
| 30421 | GTTTGCTGAG | TATAATAAAT | ACAGAAATTA | GAATCTACTG | GGGCTCCTGT | AAACCTCACC |
| 30481 | CGCCATCCTG | TGAACGCCAC | CGTTTTTACC | CACCCAAAGC | AGACCAAAGC | CTCTTCATTT |
| 30541 | TCCGGTTTGC | ACAAGCGGGC | CAATAAGTAC | CTTACCTGGT | ACTTTAAOCG | TCTCGGCTTC |
| 30601 | GTAATTTACA | ACAGTTTCCA | GCGAGACGAA | GTAAGTTTGC | CACACAACCT | GGAACGTCAG |
| 30661 | AACTACACCG | TCAAGAAAAA | CACCACCACC | ACCACCCTCC | TCACCTGCOG | CATTACTCCC |
| 30721 | AGTGCGTCAC | CGGTTGCTGC | GCCCCACACT | ACAGCCTGAG | CGTAACCAGA | AGCATTTTGC |
| 30781 | ATTTTCCCAA | AACAGGAGGT | GAGCTCAACT | CCCGGAACTC | AGGTCAAAAA | CAAGCTTGTC |
| 30841 | GGGGTGCTGG | GATTTTTTAA | TAAAGTATAT | GAGCAATTCA | AGTAACTCTA | TTATTCTTAT |
| 30901 | TAATTTTTCT | GGAAATGGGG | TCCGGGGTTAT | CCTTACTCTT | GTAATTTCTGT | CCTATTGTCA |
| 30961 | ACTAGCACTT | CTGTGCCCTA | GGGTGCGCGC | CTGCTGCACG | TGATTTTAGG | CTTGCTCGCC |
| 31021 | GCTTTTTTAA | CGCTGGGGCG | AACATCCCAAG | ATGAGGTACA | AGGAACCAGC | TTGCAATGTT |
| 31081 | CTTGCGGCAG | TCTGCAGCGC | TGCCAAAAAG | GTTGAGTTTA | AATGCACCAC | AGAACATGAA |
| 31141 | ACATTTAAAT | CAGAAGCTAA | TGAATGCACT | ACTCTTATAA | GGCAAGTATG | TATTTGGCAG |
| 31201 | AAGCTTATTA | TTGCCCACAA | AGACAAAATT | GGCAAGTATG | GTGAAAATCG | TAAAACCTTT |
| 31261 | CCAGGTGACA | CTAACGACTA | TAATGTCACA | GTCTTCCAAG | TGTACATGAG | CAAACAGTAC |
| 31321 | ATGTATATAAT | TTCCATPTTA | TGAAATGTGC | GATATTACCA | CCTTTTGTTC | CACCGCTCTG |
| 31381 | AAGTTGTGGC | CCCCACAAAA | GTGTTTAGAG | AACACTGGCA | TCAAATACAA | AAGCAGACGC |
| 31441 | CTTATTACAG | CGCTTGCTTT | GATATGTACC | TTACTTTATC | GCTTGATATC | CCCTGGACAA |
| 31501 | AGTTTPTATTG | ATGAAAAGAA | AATGCCTTGA | TTTTCGCTTT | ACCCACAACC | TTCAAATCAA |
| 31561 | TTTACTCTAT | GTGGGATATG | CTCCAGGCGG | GCAAGATTAT | CCAGCGCCTG | TTGATCAAAAC |
| 31621 | ACTTTCTTGG | ACGTTAGCGC | CTGATTTCTG | CCAGCGCCTG | CATCGCGCCC | ACAACGGACT |
| 31681 | CCAGCTTCAG | CTTGCTTGCT | CCAGAGATGA | GGACTAACAT | TTTACCCCAA | GTTTCATGCT |
| 31741 | ATCGCAACAC | CACCTGCTACC | GGACTAACAT | CTGCCCTAAA | CATAGCCCTT | ATGTTTGTTT |
| 31801 | TTGTCAATGA | CTGGGCGAGC | TTGGACATGT | GGTGGTTTTT | ACGCGCCAGA | CCCCCATCT |
| 31861 | GCCTTATTAT | TATGTGGCTT | ATTGTGTGCC | TAAAGCGCAG | TCATAGATTG | AGTCTTATA |
| 31921 | ATAGGCCTAT | CATTGTGCTC | AACCCACACA | ATGAAAAAAT | TGATTCCTCG | CGCTCACATC |
| 31981 | AACCATGTTT | TCCTCTTTTA | CAGTATGATT | AAATGAGACA | TGCGCCGGGT | CACCTTTATC |
| 32041 | TTATTGACCC | TTGTTGCGCT | TTTCTGTGCG | TGCTCTACAT | ACGGATTGTT | CTGGGTTTGT |
| 32101 | GAAGTAGATT | GCATCCCACC | TTTCACAGTT | TACCTGCTTT | AGTTTCATTGA | AGCTGATCTT |
| 32161 | CTCATCTGCA | GCCTCGTCAC | TGTAGTCATC | GCCTTCATTG | ACAGGACTAT | TTTGGCGCCC |
| 32221 | GTGCGCATTG | CGTACCTCAG | GCACCATCCG | CAATACAGAG | ACATATTTCC | GAAGCCTGGT |
| 32281 | CTCAGAAATC | TTTAAATTATG | AAACGGAGTG | TCATTTTTGT | TTTGGCGCTA | GCCATATATC |
| 32341 | TACCTGTGCT | TTGCTCCCAA | ACCTCAGGCG | CTCCCAAAAG | CGATTGTGCA | TTCCCAGTGC |
| 32401 | CTCAAATATG | GAACATTCCC | AGCTGCTACA | ACAAACAGAG | TTTGGCCCTA | CCCCCTTCTC |
| 32461 | TATACGCCAT | CATCTCTGTC | ATGGTTTTTT | GCAGTACCAT | CCACCCCTACT | |
| 32521 | CATACCTTGA | CATTGGCTGG | AATGCCATAG | ATGCCATGAA | TCAGCCTCCG | |
| 32581 | CCGCTGTGCT | ACCACTGCAA | CAGGTTATTC | CCCCAATCAA | | |

Nucleotide Sequence Analysis (cont.)

```

32641 CCACCCCCAC TGAGATTAGC TACTTTAATT TGACAGGTGG AGATGACTGA ATCTCTAGAT
32701 CTAGAATTGG ATGGAATTAA CACCGAACAG CGCCTACTAG AAAGGCGCAA GCGGGGTCC
32761 GAGCGAGAAC GCCTAAAACA AGAAGTTGAA GACATGGTTA ACCTACACCA GTGTAAAAGA
32821 GGTATCTTTT GTGTGGTCAA GCAGGCCAAA CTACCTACG AAAAAACCAC TACGGCAAC
32881 CGCCTCAGCT ACAAGCTACC CACCCAGGCG CAAAAACTGG TGCTTATGGT GGGAGAAAAA
32941 CCTATCACCG TCACCCAGCA CTCGGCAGAA ACAGAGGGCT GCCTGCACTT CCCCTATCAG
33001 GGTCCAGAGG ACCTCTGCAC TCTTATTAAA ACCATGTGTG GTATTAGAGA TCTTATTCCA
33061 TTCAACTAAC ATAAACACAC AATAAATTAC TTACTTAAAA TCAGTCAGCA AATCTTTGTC
33121 CAGCTTATTC AGCATCACCT CCTTTCCTTC CTCCCACTC TCGTATCTCA GCGGCTTTT
33181 AGCTGCAAAAC TTTCTCCAAA GTTTAAATGG GATGTCAAAT TCCTCATGTT CTGTCCCTC
33241 CGCACCCACT ATCTTCATAT TGTTGCAGAT GAAACGGGCC AGACCGTCTG AAGACACCTT
33301 CAACCCCGTG TATCCATATG ACACAGAAAC CGGGCCTCCA ACTGTGCCCT TTCTTACCCC
33361 TCCATTTGTT TCACCCAATG GTTTCGAAGA AAGTCCCCCT GGAGTCTCT CTCTAOGCGT
33421 CTCGAACCT TTGGACACCT CCCACGGCAT GCTTGGCTT AAAATGGGCA GGGGTATTAC
33481 CTTAGACAAG CCGGGAAC TCACCTCCCA AAATGTAAAC ACTGTPACTC AGCCACTTAA
33541 AAAAAAAG TCAAAACATA GTTTOGACAC CTCGCACCA CTTACAATTA CCTCAGGCGC
33601 CCTAACAGTG GCAACCACCG CTCCTCTGAT AGTTACTAGC GGGCTCTTA GGTACAGTC
33661 ACAAGCCCCA CTGACCGTGC AAGACTCCAA ACTAAGCATT GCTACTAAAG GGGCCATTAC
33721 AGTGTGAGAT GGAAAGCTAG CCCTGCAAC ATCAGCCCCC CTCTCTGGCA GTGACAGCGA
33781 CACCTTACT GTAAGTGCAT CACCCCGCT AACTACTGCC ACGGTAGCT TGGGCATTAA
33841 CATGGAAGAT CCTATTTATG TAAATAATGG AAAAATAGGA ATTAAAAATA GGGTCTTTT
33901 GCAAGTAGCA CAAAACCTCG ATACACTAAC AGTAGTTACT GGACCAGGTG TCACCGTTGA
33961 ACAAACCTCC CTTAGAACCA AAGTTGCAGG AGCTATTGGT TATGATTCAT CAAACAACAT
34021 GGAAATTAAA ACGGGCGGTG GCATGOGTAT AAATAACAAC TTGTAAATTC TAGATGTGGA
34081 TTACCCATTT GATGCTCAA CAAAACCTAG TCTTAAACTG GGGCAGGGAC CCCTGTATAT
34141 TAATGCATCT CATAACTTGG ACATAAACTA TAACAGAGGC CTATACCTTT TTAATGCATC
34201 AAACAATACT AAAAAACTGG AAGTTAGCAT AAAAAATCC AGTGGACTAA ACTTTGATAA
34261 TACTGCCATA GCTATAAATG CAGGAAAGGG TCTGGAGTTT GATACAAACA CATCTGAGTC
34321 TCCAGATATC AACCCAATAA AAACATAAAT TGGCTCTGGC ATTGATTACA ATGAAAAOOG
34381 TGCCATGATT ACTAAACTTG GAGCGGGTTT AAGCTTTGAC AACTCAGGGG CCATTACAAT
34441 AGGAAACAAA AATGATGACA AACTTACCCT GTGGACAACC CCAGACCCAT CTCCTACTG
34501 CAGAATTCAT TCAGATAATG ACTGCAAAAT TACTTTGGTT CTTACAAAAT GTGGAGTCA
34561 AGTACTAGCT CTCTGCTGT TCTTGGCTGT ATCTGGAGAT CTTTCATCCA TGACAGGCAC
34621 CGTTGCAAGT GTTAGTATAT TCCTTAGATT TGACCAAAAC GGTGTCTTAA TGGAGAACTC
34681 CTCACCTAAA AAACATTACT GGAACCTTAG AAATGGGAAC TCAACTAATG CAAATCCATA
34741 CACAAATGCA GTTGGATTTA TGCCTAACCT TCTAGCCTAT CCAAAAAACC AAAGTCAAAC
34801 TGCTAAAAAT AACATTGTCA GTCAAGTTTA CTTCATGGT GATAAAACTA AACCTATGAT
34861 ACTTACCATT ACACCTAATG GCACAGTTGA ATCCACAGAA ACTAGCGAGG TAAGCACTTA
34921 CTCTATGTCT TTTACATGGT CCTGGGAAAG TGGAAAATAC ACCACTGAAA CTTTTGCTAC
34981 CAACTCTTAC ACCTTCTCCT ACATTGCCCA GGAATAAAGA ATCGTGAACC TGTGTCATGT
35041 TATGTTTCAA CGTGGGATCC TTTATTATAG GCGAAGTCCA CGCCTACATG GGGGTAGAGT
35101 CATAATCGTG CATCAGGATA GGGCGGTGGT GCTGCAGCAG CGCGGAATA AACTGCTGCC
35161 GCGCGGCTC CGTCTGCAG GAATACAACA TGGCAGTGGT CTCTCAGCG ATGATTCCGA
35221 CCGCCCGCAG CATGAGACGC CTTGTCTCCT GGGCACAGCA GCGCACCTG ATCTCACTTA
35281 AATCAGCACA GTAAGTGCAG CACAGCACCA CAATATTGTT CAAAATCCCA CAGTGCAAGG
35341 CGCTGTATCC AAAGCTCATG GCGGGGACCA CAGAACCAC GTGGCCATCA TACCACAAGC
35401 GCAGGTAGAT TAAGTGGCGA CCCCTCATTA ACACGCTGGA CATAAACATT ACCTCTTTT
35461 GCATGTTGTA ATTACACCAC TCCCGGTACC ATATAAACCT CTGATTAAAC ATGGCGCCAT
35521 CCACCACCAT CTTAAACCAG CTGGCCAAAA CCTGCCCGCC GGCTATGCAC TGCAGGGAAC
35581 CGGGACTGGA ACAATGACAG TGGAGAGCCC AGGACTCGTA ACCATGGATC ATCATGCTCG
35641 TCATGATATC AATGTTGGCA CAACACAGGC ACACGTGCAT ACCTTCCTC AGGATTACAA
35701 GCTCTCCCG CGTCAGAAC ATATCCCAGG GAACAACCCA TTCTGAATC AGCGTAAATC
35761 CCACACTGCA GGAAGACCT CGCAGTAAC TCACGTTGTG CATGTCAAAA GTGTACATT
35821 CCGGCAGCAG CGGATGATCC TCCAGTATGG TAGCGGGGT CTCTGTCTCA AAAGGAGGTA
35881 GGCGATCCCT ACTGTACGGA GTGCGCCGAG ACAACCGAGA TCGTGTGGT CGTAGTGTCA
35941 TGCCAAATGG AACGCCGAG GTAGTCATAT TTCATCGACA CGGCACCAGC TCAATCAGTC
36001 ACAGTGTAAG AAGGGCCAAG TACAGAGCGA GTATATATAG GACTAAAAAA TGACGTAACC

```

Nucleotide Sequence Analysis (cont.)

36061 GTTAAAGTCC AAAAAAACA CCCAGAAAAC CGCAGCGGAA CCTACGCCCA GAAACGAAAG
36121 CCAAAAAACC CACAACTTCC TCAAATCTTC ACTTCGGTTC TCCCACGATA CGTCACTTCC
36181 CATTTTAAAA AACTACAAT TCCAATACA TGCAAGTTAC TCGGCCCTAA AACCTACGTC
36241 ACCCGCCCCG TTCCACGCC CCGGCCACG TCACAAACTC CACCCCTCA TTATCATATT
36301 GGCTTCAATC CAAAATAAGG TATATTATGA TGATG

//

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANTS: Gregory, R.J., Armentano, D., Couture, L.A., Smith,
A.E.

10

(ii) TITLE OF INVENTION: GENE THERAPY FOR CYSTIC FIBROSIS

(iii) NUMBER OF SEQUENCES: 9

15

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: LAHIVE & COCKFIELD
- (B) STREET: 60 STATE STREET, SUITE 510
- (C) CITY: BOSTON
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: USA
- (F) ZIP: 02109

20

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: ASCII

25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 02-DEC-1993
- (C) CLASSIFICATION:

30

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/985,478
- (B) FILING DATE: 02-DEC-1992
- (C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Hanley, Elizabeth A.
- (B) REGISTRATION NUMBER: 33,505
- (C) REFERENCE/DOCKET NUMBER: NZI-014CP2PC

40

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (617) 227-7400
- (B) TELEFAX: (617) 227-5941

45

(2) INFORMATION FOR SEQ ID NO:1:

50

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6129 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: cDNA

2145641

- 97 -

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 133..4572

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | | |
|----|------------------------------------------------------------------|-----|
| 10 | AATTGGAAGC AAATGACATC ACAGCAGGTC AGAGAAAAAG GGTGAGCGG CAGGCACCCA | 60 |
| | GAGTAGTAGG TCTTTGGCAT TAGGAGCTTG AGCCAGACG GCCCTAGCAG GGACCCACGC | 120 |
| | GCCCGAGAGA CC ATG CAG AGG TCG CCT CTG GAA AAG GCC AGC GTT GTC | 168 |
| 15 | Met Gln Arg Ser Pro Leu Glu Lys Ala Ser Val Val | |
| | 1 5 10 | |
| | TCC AAA CTT TTT TTC AGC TGG ACC AGA CCA ATT TTG AGG AAA GGA TAC | 216 |
| | Ser Lys Leu Phe Phe Ser Trp Thr Arg Pro Ile Leu Arg Lys Gly Tyr | |
| | 15 20 25 | |
| 20 | AGA CAG CGC CTG GAA TTG TCA GAC ATA TAC CAA ATC CCT TCT GTT GAT | 264 |
| | Arg Gln Arg Leu Glu Leu Ser Asp Ile Tyr Gln Ile Pro Ser Val Asp | |
| | 30 35 40 | |
| 25 | TCT GCT GAC AAT CTA TCT GAA AAA TTG GAA AGA GAA TGG GAT AGA GAG | 312 |
| | Ser Ala Asp Asn Leu Ser Glu Lys Leu Glu Arg Glu Trp Asp Arg Glu | |
| | 45 50 55 60 | |
| 30 | CTG GCT TCA AAG AAA AAT CCT AAA CTC ATT AAT GCC CTT CGG CGA TGT | 360 |
| | Leu Ala Ser Lys Lys Asn Pro Lys Leu Ile Asn Ala Leu Arg Arg Cys | |
| | 65 70 75 | |
| 35 | TTT TTC TGG AGA TTT ATG TTC TAT GGA ATC TTT TTA TAT TTA GGG GAA | 408 |
| | Phe Phe Trp Arg Phe Met Phe Tyr Gly Ile Phe Leu Tyr Leu Gly Glu | |
| | 80 85 90 | |
| | GTC ACC AAA GCA GTA CAG CCT CTC TTA CTG GGA AGA ATC ATA GCT TCC | 456 |
| | Val Thr Lys Ala Val Gln Pro Leu Leu Glu Gly Arg Ile Ile Ala Ser | |
| | 95 100 105 | |
| 40 | TAT GAC CCG GAT AAC AAG GAG GAA CGC TCT ATC GCG ATT TAT CTA GGC | 504 |
| | Tyr Asp Pro Asp Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly | |
| | 110 115 120 | |
| 45 | ATA GGC TTA TGC CTT CTC TTT ATT GTG AGG ACA CTG CTC CTA CAC CCA | 552 |
| | Ile Gly Leu Cys Leu Leu Phe Ile Val Arg Thr Leu Leu Leu His Pro | |
| | 125 130 135 140 | |
| 50 | GCC ATT TTT GGC CTT CAT CAC ATT GGA ATG CAG ATG AGA ATA GCT ATG | 600 |
| | Ala Ile Phe Gly Leu His His Ile Gly Met Gln Met Arg Ile Ala Met | |
| | 145 150 155 | |
| 55 | TTT AGT TTG ATT TAT AAG AAG ACT TTA AAG CTG TCA AGC CGT GTT CTA | 648 |
| | Phe Ser Leu Ile Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu | |
| | 160 165 170 | |

| | | |
|----|-----------------------------------------------------------------|------|
| | GAT AAA ATA AGT ATT GGA CAA CTT GTT AGT CTC CTT TCC AAC AAC CTG | 696 |
| | Asp Lys Ile Ser Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu | |
| | 175 180 185 | |
| 5 | AAC AAA TTT GAT GAA GGA CTT GCA TTG GCA CAT TTC GTG TGG ATC GCT | 744 |
| | Asn Lys Phe Asp Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala | |
| | 190 195 200 | |
| 10 | CCT TTG CAA GTG GCA CTC CTC ATG GGG CTA ATC TGG GAG TTG TTA CAG | 792 |
| | Pro Leu Gln Val Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln | |
| | 205 210 215 220 | |
| 15 | GCG TCT GCC TTC TGT GGA CTT GGT TTC CTG ATA GTC CTT GCC CTT TTT | 840 |
| | Ala Ser Ala Phe Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe | |
| | 225 230 235 | |
| 20 | CAG GCT GGG CTA GGG AGA ATG ATG ATG AAG TAC AGA GAT CAG AGA GCT | 888 |
| | Gln Ala Gly Leu Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala | |
| | 240 245 250 | |
| 25 | GGG AAG ATC AGT GAA AGA CTT GTG ATT ACC TCA GAA ATG ATT GAA AAT | 936 |
| | Gly Lys Ile Ser Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn | |
| | 255 260 265 | |
| 30 | ATC CAA TCT GTT AAG GCA TAC TGC TGG GAA GAA GCA ATG GAA AAA ATG | 984 |
| | Ile Gln Ser Val Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met | |
| | 270 275 280 | |
| 35 | ATT GAA AAC TTA AGA CAA ACA GAA CTG AAA CTG ACT CGG AAG GCA GCC | 1032 |
| | Ile Glu Asn Leu Arg Gln Thr Glu Leu Lys Leu Thr Arg Lys Ala Ala | |
| | 285 290 295 300 | |
| 40 | TAT GTG AGA TAC TTC AAT AGC TCA GCC TTC TTC TTC TCA GGG TTC TTT | 1080 |
| | Tyr Val Arg Tyr Phe Asn Ser Ser Ala Phe Phe Phe Ser Gly Phe Phe | |
| | 305 310 315 | |
| 45 | GTG GTG TTT TTA TCT GTG CTT CCC TAT GCA CTA ATC AAA GGA ATC ATC | 1128 |
| | Val Val Phe Leu Ser Val Leu Pro Tyr Ala Leu Ile Lys Gly Ile Ile | |
| | 320 325 330 | |
| 50 | CTC CGG AAA ATA TTC ACC ACC ATC TCA TTC TGC ATT GTT CTG CGC ATG | 1176 |
| | Leu Arg Lys Ile Phe Thr Thr Ile Ser Phe Cys Ile Val Leu Arg Met | |
| | 335 340 345 | |
| 55 | GCG GTC ACT CGG CAA TTT CCC TGG GCT GTA CAA ACA TGG TAT GAC TCT | 1224 |
| | Ala Val Thr Arg Gln Phe Pro Trp Ala Val Gln Thr Trp Tyr Asp Ser | |
| | 350 355 360 | |
| 60 | CTT GGA GCA ATA AAC AAA ATA CAG GAT TTC TTA CAA AAG CAA GAA TAT | 1272 |
| | Leu Gly Ala Ile Asn Lys Ile Gln Asp Phe Leu Gln Lys Gln Glu Tyr | |
| | 365 370 375 380 | |
| 65 | AAG ACA TTG GAA TAT AAC TTA ACG ACT ACA GAA GTA GTG ATG GAG AAT | 1320 |
| | Lys Thr Leu Glu Tyr Asn Leu Thr Thr Thr Glu Val Val Met Glu Asn | |
| | 385 390 395 | |

| | | | | | | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| | GTA | ACA | GCC | TTC | TGG | GAG | GAG | GGA | TTT | GGG | GAA | TTA | TTT | GAG | AAA | GCA | 1368 |
| | Val | Thr | Ala | Phe | Trp | Glu | Glu | Gly | Phe | Gly | Glu | Leu | Phe | Glu | Lys | Ala | |
| | | | | 400 | | | | | 405 | | | | | 410 | | | |
| 5 | AAA | CAA | AAC | AAT | AAC | AAT | AGA | AAA | ACT | TCT | AAT | GGT | GAT | GAC | AGC | CTC | 1416 |
| | Lys | Gln | Asn | Asn | Asn | Asn | Arg | Lys | Thr | Ser | Asn | Gly | Asp | Asp | Ser | Leu | |
| | | | 415 | | | | | 420 | | | | | 425 | | | | |
| 10 | TTC | TTC | AGT | AAT | TTC | TCA | CTT | CTT | GGT | ACT | GCT | GTC | CTG | AAA | GAT | ATT | 1464 |
| | Phe | Phe | Ser | Asn | Phe | Ser | Leu | Leu | Gly | Thr | Pro | Val | Leu | Lys | Asp | Ile | |
| | | | 430 | | | | 435 | | | | | 440 | | | | | |
| 15 | AAT | TTC | AAG | ATA | GAA | AGA | GGA | CAG | TTG | TTG | GCG | GTT | GCT | GGA | TCC | ACT | 1512 |
| | Asn | Phe | Lys | Ile | Glu | Arg | Gly | Gln | Leu | Leu | Ala | Val | Ala | Gly | Ser | Thr | |
| | 445 | | | | | 450 | | | | | 455 | | | | | 460 | |
| 20 | GGA | GCA | GGC | AAG | ACT | TCA | CTT | CTA | ATG | ATG | ATT | ATG | GGA | GAA | CTG | GAG | 1560 |
| | Gly | Ala | Gly | Lys | Thr | Ser | Leu | Leu | Met | Met | Ile | Met | Gly | Glu | Leu | Glu | |
| | | | | | 465 | | | | | 470 | | | | | 475 | | |
| 25 | CCT | TCA | GAG | GGT | AAA | ATT | AAG | CAC | AGT | GGA | AGA | ATT | TCA | TTC | TGT | TCT | 1608 |
| | Pro | Ser | Glu | Gly | Lys | Ile | Lys | His | Ser | Gly | Arg | Ile | Ser | Phe | Cys | Ser | |
| | | | | 480 | | | | | 485 | | | | | | 490 | | |
| 30 | CAG | TTT | TCC | TGG | ATT | ATG | CCT | GGC | ACC | ATT | AAA | GAA | AAT | ATC | ATC | TTT | 1656 |
| | Gln | Phe | Ser | Trp | Ile | Met | Pro | Gly | Thr | Ile | Lys | Glu | Asn | Ile | Ile | Phe | |
| | | | | 495 | | | | 500 | | | | | 505 | | | | |
| 35 | GGT | GTT | TCC | TAT | GAT | GAA | TAT | AGA | TAC | AGA | AGC | GTC | ATC | AAA | GCA | TGC | 1704 |
| | Gly | Val | Ser | Tyr | Asp | Glu | Tyr | Arg | Tyr | Arg | Ser | Val | Ile | Lys | Ala | Cys | |
| | | | | 510 | | | | 515 | | | | 520 | | | | | |
| 40 | CAA | CTA | GAA | GAG | GAC | ATC | TCC | AAG | TTT | GCA | GAG | AAA | GAC | AAT | ATA | GTT | 1752 |
| | Gln | Leu | Glu | Glu | Asp | Ile | Ser | Lys | Phe | Ala | Glu | Lys | Asp | Asn | Ile | Val | |
| | 525 | | | | | 530 | | | | | 535 | | | | | 540 | |
| 45 | CTT | GGA | GAA | GGT | GGA | ATC | ACA | CTG | AGT | GGA | GGT | CAA | CGA | GCA | AGA | ATT | 1800 |
| | Leu | Gly | Glu | Gly | Gly | Ile | Thr | Leu | Ser | Gly | Gly | Gln | Arg | Ala | Arg | Ile | |
| | | | | | 545 | | | | | 550 | | | | | 555 | | |
| 50 | TCT | TTA | GCA | AGA | GCA | GTA | TAC | AAA | GAT | GCT | GAT | TTG | TAT | TTA | TTA | GAC | 1848 |
| | Ser | Leu | Ala | Arg | Ala | Val | Tyr | Lys | Asp | Ala | Asp | Leu | Tyr | Leu | Leu | Asp | |
| | | | | 560 | | | | | 565 | | | | | 570 | | | |
| 55 | TCT | CCT | TTT | GGA | TAC | CTA | GAT | GTT | TTA | ACA | GAA | AAA | GAA | ATA | TTT | GAA | 1896 |
| | Ser | Pro | Phe | Gly | Tyr | Leu | Asp | Val | Leu | Thr | Glu | Lys | Glu | Ile | Phe | Glu | |
| | | | | 575 | | | | 580 | | | | | 585 | | | | |
| 60 | AGC | TGT | GTC | TGT | AAA | CTG | ATG | GCT | AAC | AAA | ACT | AGG | ATT | TTG | GTC | ACT | 1944 |
| | Ser | Cys | Val | Cys | Lys | Leu | Met | Ala | Asn | Lys | Thr | Arg | Ile | Leu | Val | Thr | |
| | | | | 590 | | | | 595 | | | | 600 | | | | | |
| 65 | TCT | AAA | ATG | GAA | CAT | TTA | AAG | AAA | GCT | GAC | AAA | ATA | TTA | ATT | TTG | CAT | 1992 |
| | Ser | Lys | Met | Glu | His | Leu | Lys | Lys | Ala | Asp | Lys | Ile | Leu | Ile | Leu | His | |
| | 605 | | | | | 610 | | | | | 615 | | | | | 620 | |

| | | |
|----|-----------------------------------------------------------------|------|
| | GAA GGT AGC AGC TAT TTT TAT GGG ACA TTT TCA GAA CTC CAA AAT CTA | 2040 |
| | Glu Gly Ser Ser Tyr Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu | |
| | 625 630 635 | |
| 5 | CAG CCA GAC TTT AGC TCA AAA CTC ATG GGA TGT GAT TCT TTC GAC CAA | 2088 |
| | Gln Pro Asp Phe Ser Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln | |
| | 640 645 650 | |
| 10 | TTT AGT GCA GAA AGA AGA AAT TCA ATC CTA ACT GAG ACC TTA CAC CGT | 2136 |
| | Phe Ser Ala Glu Arg Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg | |
| | 655 660 665 | |
| 15 | TTC TCA TTA GAA GGA GAT GCT CCT GTC TCC TGG ACA GAA ACA AAA AAA | 2184 |
| | Phe Ser Leu Glu Gly Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys | |
| | 670 675 680 | |
| 20 | CAA TCT TTT AAA CAG ACT GGA GAG TTT GGG GAA AAA AGG AAG AAT TCT | 2232 |
| | Gln Ser Phe Lys Gln Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser | |
| | 685 690 695 700 | |
| 25 | ATT CTC AAT CCA ATC AAC TCT ATA CGA AAA TTT TCC ATT GTG CAA AAG | 2280 |
| | Ile Leu Asn Pro Ile Asn Ser Ile Arg Lys Phe Ser Ile Val Gln Lys | |
| | 705 710 715 | |
| 30 | ACT CCC TTA CAA ATG AAT GGC ATC GAA GAG GAT TCT GAT GAG CCT TTA | 2328 |
| | Thr Pro Leu Gln Met Asn Gly Ile Glu Glu Asp Ser Asp Glu Pro Leu | |
| | 720 725 730 | |
| 35 | GAG AGA AGG CTG TCC TTA GTA CCA GAT TCT GAG CAG GGA GAG GCG ATA | 2376 |
| | Glu Arg Arg Leu Ser Leu Val Pro Asp Ser Glu Gln Gly Glu Ala Ile | |
| | 735 740 745 | |
| 40 | CTG CCT CGC ATC AGC GTG ATC AGC ACT GGC CCC ACG CTT CAG GCA CGA | 2424 |
| | Leu Pro Arg Ile Ser Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg | |
| | 750 755 760 | |
| 45 | AGG AGG CAG TCT GTC CTG AAC CTG ATG ACA CAC TCA GTT AAC CAA GGT | 2472 |
| | Arg Arg Gln Ser Val Leu Asn Leu Met Thr His Ser Val Asn Gln Gly | |
| | 765 770 775 780 | |
| 50 | CAG AAC ATT CAC CGA AAG ACA ACA GCA TCC ACA CGA AAA GTG TCA CTG | 2520 |
| | Gln Asn Ile His Arg Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu | |
| | 785 790 795 | |
| 55 | GCC CCT CAG GCA AAC TTG ACT GAA CTG GAT ATA TAT TCA AGA AGG TTA | 2568 |
| | Ala Pro Gln Ala Asn Leu Thr Glu Leu Asp Ile Tyr Ser Arg Arg Leu | |
| | 800 805 810 | |
| 60 | TCT CAA GAA ACT GGC TTG GAA ATA AGT GAA GAA ATT AAC GAA GAA GAC | 2616 |
| | Ser Gln Glu Thr Gly Leu Glu Ile Ser Glu Glu Ile Asn Glu Glu Asp | |
| | 815 820 825 | |
| 65 | TTA AAG GAG TGC CTT TTT GAT GAT ATG GAG AGC ATA CCA GCA GTG ACT | 2664 |
| | Leu Lys Glu Cys Leu Phe Asp Asp Met Glu Ser Ile Pro Ala Val Thr | |
| | 830 835 840 | |

2145641

- 101 -

| | | | | | | | | | | | | | | | | | |
|----|-----|------|------|------|------|------|-----|------|-----|------|------|-----|------|------|------|------|------|
| | ACA | TGG | AAC | ACA | TAC | CTT | CGA | TAT | ATT | ACT | GTC | CAC | AAG | AGC | TTA | ATT | 2712 |
| | Thr | Trp | Asn | Thr | Tyr | Leu | Arg | Tyr | Ile | Thr | Val | His | Lys | Ser | Leu | Ile | |
| | 845 | | | | | 850 | | | | | 855 | | | | | 860 | |
| 5 | TTT | GTG | CTA | ATT | TGG | TGC | TTA | GTA | ATT | TTT | CTG | GCA | GAG | GTG | GCT | GCT | 2760 |
| | Phe | Val | Leu | Ile | Trp | Cys | Leu | Val | Ile | Phe | Leu | Ala | Glu | Val | Ala | Ala | |
| | | | | | 865 | | | | | 870 | | | | | | 875 | |
| 10 | TCT | TTG | GTT | GTG | CTG | TGG | CTC | CTT | GGA | AAC | ACT | CCT | CTT | CAA | GAC | AAA | 2808 |
| | Ser | Leu | Val | Val | Leu | Trp | Leu | Leu | Gly | Asn | Thr | Pro | Leu | Gln | Asp | Lys | |
| | | | | | 880 | | | | 885 | | | | | 890 | | | |
| 15 | GGG | AAT | AGT | ACT | CAT | AGT | AGA | AAT | AAC | AGC | TAT | GCA | GTG | ATT | ATC | ACC | 2856 |
| | Gly | Asn | Ser | Thr | His | Ser | Arg | Asn | Asn | Ser | Tyr | Ala | Val | Ile | Ile | Thr | |
| | | | 895 | | | | | 900 | | | | | | 905 | | | |
| 20 | AGC | ACC | AGT | TCG | TAT | TAT | GTG | TTT | TAC | ATT | TAC | GTG | GGA | GTA | GCC | GAC | 2904 |
| | Ser | Thr | Ser | Ser | Tyr | Tyr | Val | Phe | Tyr | Ile | Tyr | Val | Gly | Val | Ala | Asp | |
| | | 910 | | | | | 915 | | | | | | 920 | | | | |
| 25 | ACT | TTG | CTT | GCT | ATG | GGA | TTC | TTC | AGA | GGT | CTA | CCA | CTG | GTG | CAT | ACT | 2952 |
| | Thr | Leu | Leu | Ala | Met | Gly | Phe | Phe | Arg | Gly | Leu | Pro | Leu | Val | His | Thr | |
| | | 925 | | | | 930 | | | | | 935 | | | | | 940 | |
| 30 | CTA | ATC | ACA | GTG | TCG | AAA | ATT | TTA | CAC | CAC | AAA | ATG | TTA | CAT | TCT | GTT | 3000 |
| | Leu | Ile | Thr | Val | Ser | Lys | Ile | Leu | His | His | Lys | Met | Leu | His | Ser | Val | |
| | | | | | 945 | | | | | 950 | | | | | 955 | | |
| 35 | CTT | CAA | GCA | CCT | ATG | TCA | ACC | CTC | AAC | ACG | TTG | AAA | GCA | GGT | GGG | ATT | 3048 |
| | Leu | Gln | Ala | Pro | Met | Ser | Thr | Leu | Asn | Thr | Leu | Lys | Ala | Gly | Gly | Ile | |
| | | | | 960 | | | | | 965 | | | | | 970 | | | |
| 40 | CTT | AAT | AGA | TTC | TCC | AAA | GAT | ATA | GCA | ATT | TTG | GAT | GAC | CTT | CTG | CCT | 3096 |
| | Leu | Asn | Arg | Phe | Ser | Lys | Asp | Ile | Ala | Ile | Leu | Asp | Asp | Leu | Leu | Pro | |
| | | | 975 | | | | | 980 | | | | | 985 | | | | |
| 45 | CTT | ACC | ATA | TTT | GAC | TTC | ATC | CAG | TTG | TTA | TTA | ATT | GTG | ATT | GGA | GCT | 3144 |
| | Leu | Thr | Ile | Phe | Asp | Phe | Ile | Gln | Leu | Leu | Leu | Ile | Val | Ile | Gly | Ala | |
| | | 990 | | | | | 995 | | | | | | 1000 | | | | |
| 50 | ATA | GCA | GTT | GTC | GCA | GTT | TTA | CAA | CCC | TAC | ATC | TTT | GTT | GCA | ACA | GTG | 3192 |
| | Ile | Ala | Val | Val | Ala | Val | Leu | Gln | Pro | Tyr | Ile | Phe | Val | Ala | Thr | Val | |
| | | 1005 | | | | 1010 | | | | | 1015 | | | | | 1020 | |
| 55 | CCA | GTG | ATA | GTG | GCT | TTT | ATT | ATG | TTG | AGA | GCA | TAT | TTC | CTC | CAA | ACC | 3240 |
| | Pro | Val | Ile | Val | Ala | Phe | Ile | Met | Leu | Arg | Ala | Tyr | Phe | Leu | Gln | Thr | |
| | | | | | 1025 | | | | | 1030 | | | | | 1035 | | |
| 60 | TCA | CAG | CAA | CTC | AAA | CAA | CTG | GAA | TCT | GAA | GGC | AGG | AGT | CCA | ATT | TTC | 3288 |
| | Ser | Gln | Gln | Leu | Lys | Gln | Leu | Glu | Ser | Glu | Gly | Arg | Ser | Pro | Ile | Phe | |
| | | | | 1040 | | | | 1045 | | | | | | 1050 | | | |
| 65 | ACT | CAT | CTT | GTT | ACA | AGC | TTA | AAA | GGA | CTA | TGG | ACA | CTT | CGT | GCC | TTC | 3336 |
| | Thr | His | Leu | Val | Thr | Ser | Leu | Lys | Gly | Leu | Trp | Thr | Leu | Arg | Ala | Phe | |
| | | | 1055 | | | | | 1060 | | | | | | 1065 | | | |

| | | |
|----|-----------------------------------------------------------------|------|
| | GGA CGG CAG CCT TAC TTT GAA ACT CTG TTC CAC AAA GCT CTG AAT TTA | 3384 |
| | Gly Arg Gln Pro Tyr Phe Glu Thr Leu Phe His Lys Ala Leu Asn Leu | |
| | 1070 1075 1080 | |
| 5 | CAT ACT GCC AAC TGG TTC TTG TAC CTG TCA ACA CTG CGC TGG TTC CAA | 3432 |
| | His Thr Ala Asn Trp Phe Leu Tyr Leu Ser Thr Leu Arg Trp Phe Gln | |
| | 1085 1090 1095 1100 | |
| 10 | ATG AGA ATA GAA ATG ATT TTT GTC ATC TTC TTC ATT GCT GTT ACC TTC | 3480 |
| | Met Arg Ile Glu Met Ile Phe Val Ile Phe Phe Ile Ala Val Thr Phe | |
| | 1105 1110 1115 | |
| 15 | ATT TCC ATT TTA ACA ACA GGA GAA GGA GAA GGA AGA GTT GGT ATT ATC | 3528 |
| | Ile Ser Ile Leu Thr Thr Gly Glu Gly Glu Gly Arg Val Gly Ile Ile | |
| | 1120 1125 1130 | |
| 20 | CTG ACT TTA GCC ATG AAT ATC ATG AGT ACA TTG CAG TGG GCT GTA AAC | 3576 |
| | Leu Thr Leu Ala Met Asn Ile Met Ser Thr Leu Gln Trp Ala Val Asn | |
| | 1135 1140 1145 | |
| 25 | TCC AGC ATA GAT GTG GAT AGC TTG ATG CGA TCT GTG AGC CGA GTC TTT | 3624 |
| | Ser Ser Ile Asp Val Asp Ser Leu Met Arg Ser Val Ser Arg Val Phe | |
| | 1150 1155 1160 | |
| 30 | AAG TTC ATT GAC ATG CCA ACA GAA GGT AAA CCT ACC AAG TCA ACC AAA | 3672 |
| | Lys Phe Ile Asp Met Pro Thr Glu Gly Lys Pro Thr Lys Ser Thr Lys | |
| | 1165 1170 1175 1180 | |
| 35 | CCA TAC AAG AAT GGC CAA CTC TCG AAA GTT ATG ATT ATT GAG AAT TCA | 3720 |
| | Pro Tyr Lys Asn Gly Gln Leu Ser Lys Val Met Ile Ile Glu Asn Ser | |
| | 1185 1190 1195 | |
| 40 | CAC GTG AAG AAA GAT GAC ATC TGG CCC TCA GGG GGC CAA ATG ACT GTC | 3768 |
| | His Val Lys Lys Asp Asp Ile Trp Pro Ser Gly Gly Gln Met Thr Val | |
| | 1200 1205 1210 | |
| 45 | AAA GAT CTC ACA GCA AAA TAC ACA GAA GGT GGA AAT GCC ATA TTA GAG | 3816 |
| | Lys Asp Leu Thr Ala Lys Tyr Thr Glu Gly Gly Asn Ala Ile Leu Glu | |
| | 1215 1220 1225 | |
| 50 | AAC ATT TCC TTC TCA ATA AGT CCT GGC CAG AGG GTG GGC CTC TTG GGA | 3864 |
| | Asn Ile Ser Phe Ser Ile Ser Pro Gly Gln Arg Val Gly Leu Leu Gly | |
| | 1230 1235 1240 | |
| 55 | AGA ACT GGA TCA GGG AAG AGT ACT TTG TTA TCA GCT TTT TTG AGA CTA | 3912 |
| | Arg Thr Gly Ser Gly Lys Ser Thr Leu Leu Ser Ala Phe Leu Arg Leu | |
| | 1245 1250 1255 1260 | |
| 50 | CTG AAC ACT GAA GGA GAA ATC CAG ATC GAT GGT GTG TCT TGG GAT TCA | 3960 |
| | Leu Asn Thr Glu Gly Glu Ile Gln Ile Asp Gly Val Ser Trp Asp Ser | |
| | 1265 1270 1275 | |
| 55 | ATA ACT TTG CAA CAG TGG AGG AAA GCC TTT GGA GTG ATA CCA CAG AAA | 4008 |
| | Ile Thr Leu Gln Gln Trp Arg Lys Ala Phe Gly Val Ile Pro Gln Lys | |
| | 1280 1285 1290 | |

2145641

- 103 -

| | | |
|----|----------------------------------------------------------------------------------------|------|
| 5 | GTA TTT ATT TTT TCT GGA ACA TTT AGA AAA AAC TTG GAT CCC TAT GAA | 4056 |
| | Val Phe Ile Phe Ser Gly Thr Phe Arg Lys Asn Leu Asp Pro Tyr Glu 1295 1300 1305 | |
| 10 | CAG TGG AGT GAT CAA GAA ATA TGG AAA GTT GCA GAT GAG GTT GGG CTC | 4104 |
| | Gln Trp Ser Asp Gln Glu Ile Trp Lys Val Ala Asp Glu Val Gly Leu 1310 1315 1320 | |
| 15 | AGA TCT GTG ATA GAA CAG TTT CCT GGG AAG CTT GAC TTT GTC CTT GTG | 4152 |
| | Arg Ser Val Ile Glu Gln Phe Pro Gly Lys Leu Asp Phe Val Leu Val 1325 1330 1335 1340 | |
| 20 | GAT GGG GGC TGT GTC CTA AGC CAT GGC CAC AAG CAG TTG ATG TGC TTG | 4200 |
| | Asp Gly Gly Cys Val Leu Ser His Gly His Lys Gln Leu Met Cys Leu 1345 1350 1355 | |
| 25 | GCT AGA TCT GTT CTC AGT AAG GCG AAG ATC TTG CTG CTT GAT GAA CCC | 4248 |
| | Ala Arg Ser Val Leu Ser Lys Ala Lys Ile Leu Leu Leu Asp Glu Pro 1360 1365 1370 | |
| 30 | AGT GCT CAT TTG GAT CCA GTA ACA TAC CAA ATA ATT AGA AGA ACT CTA | 4296 |
| | Ser Ala His Leu Asp Pro Val Thr Tyr Gln Ile Ile Arg Arg Thr Leu 1375 1380 1385 | |
| 35 | AAA CAA GCA TTT GCT GAT TGC ACA GTA ATT CTC TGT GAA CAC AGG ATA | 4344 |
| | Lys Gln Ala Phe Ala Asp Cys Thr Val Ile Leu Cys Glu His Arg Ile 1390 1395 1400 | |
| 40 | GAA GCA ATG CTG GAA TGC CAA CAA TTT TTG GTC ATA GAA GAG AAC AAA | 4392 |
| | Glu Ala Met Leu Glu Cys Gln Gln Phe Leu Val Ile Glu Glu Asn Lys 1405 1410 1415 1420 | |
| 45 | GTG CGG CAG TAC GAT TCC ATC CAG AAA CTG CTG AAC GAG AGG AGC CTC | 4440 |
| | Val Arg Gln Tyr Asp Ser Ile Gln Lys Leu Leu Asn Glu Arg Ser Leu 1425 1430 1435 | |
| 50 | TTC CGG CAA GCC ATC AGC CCC TCC GAC AGG GTG AAG CTC TTT CCC CAC | 4488 |
| | Phe Arg Gln Ala Ile Ser Pro Ser Asp Arg Val Lys Leu Phe Pro His 1440 1445 1450 | |
| 55 | CGG AAC TCA AGC AAG TGC AAG TCT AAG CCC CAG ATT GCT GCT CTG AAA | 4536 |
| | Arg Asn Ser Ser Lys Cys Lys Ser Lys Pro Gln Ile Ala Ala Leu Lys 1455 1460 1465 | |
| 60 | GAG GAG ACA GAA GAA GAG GTG CAA GAT ACA AGG CTT TAGAGAGCAG | 4582 |
| | Glu Glu Thr Glu Glu Glu Val Gln Asp Thr Arg Leu 1470 1475 1480 | |
| 65 | CATAAATGTT GACATGGGAC ATTTGCTCAT GGAATTGGAG CTCGTGGGAC AGTCACCTCA | 4642 |
| | TGGAATTGGA GCTCGTGGAA CAGTTACCTC TGCCTCAGAA AACAAGGATG AATTAAGTTT | 4702 |
| 70 | TTTTTTAAAA AAGAAACATT TG GTAAGGGG AATTGAGGAC ACTGATATGG GTCTTGATAA | 4762 |
| | ATGGCTTCCT GGCAATAGTC AAATTGTGTG AAAGGTACTT CAAATCCTTG AAGATTACC | 4822 |
| 75 | ACTTGTGTTT TGCAAGCCAG ATTTTCCTGA AAACCCTTGC CATGTGCTAG TAATTGAAAA | 4882 |
| | | |

- 104 -

GGCAGCTCTA AATGTCAATC AGCCTAGTTG ATCAGCTTAT TGTCTAGTGA AACTCGTTAA 4942
TTTGTAGTGT TGGAGAAGAA CTGAAATCAT ACTTCTTAGG GTTATGATTA AGTAATGATA 5002
5 ACTGGAAACT TCAGCGGTTT ATATAAGCTT GTATTCCTTT TTCTCTCCTC TCCCCATGAT 5062
GTTTAGAAAC ACAACTATAT TGTTTGCTAA GCATTCCAAC TATCTCATTT CCAAGCAAGT 5122
ATTAGAATAC CACAGGAACC ACAAGACTGC ACATCAAAAT ATGCCCCATT CAACATCTAG 5182
10 TGAGCAGTCA GGAAAGAGAA CTTCCAGATC CTGGAAATCA GGGTTAGTAT TGTCCAGGTC 5242
TACCAAAAAT CTCAATATTT CAGATAATCA CAATACATCC CTTACCTGGG AAAGGGCTGT 5302
15 TATAATCTTT CACAGGGGAC AGGATGGTTC CCTTGATGAA GAAGTTGATA TGCCTTTTCC 5362
CAACTCCAGA AAGTGACAAG CTCACAGACC TTTGAACTAG AGTTTAGCTG GAAAAGTATG 5422
TTAGTGCAAA TTGTCACAGG ACAGCCCTTC TTTCCACAGA AGCTCCAGGT AGAGGGTGTG 5482
20 TAAGTAGATA GGCCATGGGC ACTGTGGGTA GACACACATG AAGTCCAAGC ATTTAGATGT 5542
ATAGGTTGAT GGTGGTATGT TTTCAGGCTA GATGTATGTA CTTCATGCTG TCTACACTAA 5602
25 GAGAGAATGA GAGACACACT GAAGAAGCAC CAATCATGAA TTAGTTTTAT ATGCTTCTGT 5662
TTTATAATTT TGTGAAGCAA AATTTTTTCT CTAGGAAATA TTTATTTTAA TAATGTTTCA 5722
AACATATATT ACAATGCTGT ATTTTAAAAG AATGATTATG AATTACATTT GTATAAAATA 5782
30 ATTTTTATAT TTGAAATATT GACTTTTTAT GGCACATGTA TTTTATGAA ATATTATGTT 5842
AAAACGGGA CAGGGGAGAA CCTAGGGTGA TATTAACCAG GGGCCATGAA TCACCTTTTG 5902
35 GTCTGGAGGG AAGCCTTGGG GCTGATCGAG TTGTTGCCCA CAGCTGTATG ATTCCAGGCC 5962
AGACACAGCC TCTTAGATGC AGTTCTGAAG AAGATGGTAC CACCAGTCTG ACTGTTTCCA 6022
TCAAGGGTAC ACTGCCTTCT CAACTCCAAA CTGACTCTTA AGAAGACTGC ATTATATTTA 6082
40 TTACTGTAAG AAAATATCAC TTGTCAATAA AATCCATACA TTTGTGT 6129

(2) INFORMATION FOR SEQ ID NO:2:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1480 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

55

Met Gln Arg Ser Pro Leu Glu Lys Ala Ser Val Val Ser Lys Leu Phe
1 5 10 15

2145641

- 105 -

Phe Ser Trp Thr Arg Pro Ile Leu Arg Lys Gly Tyr Arg Gln Arg Leu
 20 25 30

5 Glu Leu Ser Asp Ile Tyr Gln Ile Pro Ser Val Asp Ser Ala Asp Asn
 35 40 45

Leu Ser Glu Lys Leu Glu Arg Glu Trp Asp Arg Glu Leu Ala Ser Lys
 50 55 60

10 Lys Asn Pro Lys Leu Ile Asn Ala Leu Arg Arg Cys Phe Phe Trp Arg
 65 70 75 80

Phe Met Phe Tyr Gly Ile Phe Leu Tyr Leu Gly Glu Val Thr Lys Ala
 15 85 90 95

Val Gln Pro Leu Leu Leu Gly Arg Ile Ile Ala Ser Tyr Asp Pro Asp
 100 105 110

20 Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly Ile Gly Leu Cys
 115 120 125

Leu Leu Phe Ile Val Arg Thr Leu Leu Leu His Pro Ala Ile Phe Gly
 130 135 140

25 Leu His His Ile Gly Met Gln Met Arg Ile Ala Met Phe Ser Leu Ile
 145 150 155 160

Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu Asp Lys Ile Ser
 30 165 170 175

Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu Asn Lys Phe Asp
 180 185 190

35 Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala Pro Leu Gln Val
 195 200 205

Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln Ala Ser Ala Phe
 210 215 220

40 Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe Gln Ala Gly Leu
 225 230 235 240

Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala Gly Lys Ile Ser
 45 245 250 255

Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn Ile Gln Ser Val
 260 265 270

50 Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met Ile Glu Asn Leu
 275 280 285

Arg Gln Thr Glu Leu Lys Leu Thr Arg Lys Ala Ala Tyr Val Arg Tyr
 290 295 300

55 Phe Asn Ser Ser Ala Phe Phe Phe Ser Gly Phe Phe Val Val Phe Leu
 305 310 315 320

Ser Val Leu Pro Tyr Ala Leu Ile Lys Gly Ile Ile Leu Arg Lys Ile
 325 330 335

5 Phe Thr Thr Ile Ser Phe Cys Ile Val Leu Arg Met Ala Val Thr Arg
 340 345 350

Gln Phe Pro Trp Ala Val Gln Thr Trp Tyr Asp Ser Leu Gly Ala Ile
 355 360 365

10 Asn Lys Ile Gln Asp Phe Leu Gln Lys Gln Glu Tyr Lys Thr Leu Glu
 370 375 380

Tyr Asn Leu Thr Thr Thr Glu Val Val Met Glu Asn Val Thr Ala Phe
 15 385 390 395 400

Trp Glu Glu Gly Phe Gly Glu Leu Phe Glu Lys Ala Lys Gln Asn Asn
 405 410 415

20 Asn Asn Arg Lys Thr Ser Asn Gly Asp Asp Ser Leu Phe Phe Ser Asn
 420 425 430

Phe Ser Leu Leu Gly Thr Pro Val Leu Lys Asp Ile Asn Phe Lys Ile
 435 440 445

25 Glu Arg Gly Gln Leu Leu Ala Val Ala Gly Ser Thr Gly Ala Gly Lys
 450 455 460

Thr Ser Leu Leu Met Met Ile Met Gly Glu Leu Glu Pro Ser Glu Gly
 30 465 470 475 480

Lys Ile Lys His Ser Gly Arg Ile Ser Phe Cys Ser Gln Phe Ser Trp
 485 490 495

35 Ile Met Pro Gly Thr Ile Lys Glu Asn Ile Ile Phe Gly Val Ser Tyr
 500 505 510

Asp Glu Tyr Arg Tyr Arg Ser Val Ile Lys Ala Cys Gln Leu Glu Glu
 515 520 525

40 Asp Ile Ser Lys Phe Ala Glu Lys Asp Asn Ile Val Leu Gly Glu Gly
 530 535 540

Gly Ile Thr Leu Ser Gly Gly Gln Arg Ala Arg Ile Ser Leu Ala Arg
 45 545 550 555 560

Ala Val Tyr Lys Asp Ala Asp Leu Tyr Leu Leu Asp Ser Pro Phe Gly
 565 570 575

50 Tyr Leu Asp Val Leu Thr Glu Lys Glu Ile Phe Glu Ser Cys Val Cys
 580 585 590

Lys Leu Met Ala Asn Lys Thr Arg Ile Leu Val Thr Ser Lys Met Glu
 595 600 605

55 His Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu His Glu Gly Ser Ser
 610 615 620

2145641

- 107 -

Tyr Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu Gln Pro Asp Phe
 625 630 635 640
 5 Ser Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln Phe Ser Ala Glu
 645 650 655
 Arg Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg Phe Ser Leu Glu
 660 665 670
 10 Gly Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys Gln Ser Phe Lys
 675 680 685
 Gln Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser Ile Leu Asn Pro
 15 690 695 700
 Ile Asn Ser Ile Arg Lys Phe Ser Ile Val Gln Lys Thr Pro Leu Gln
 705 710 715 720
 20 Met Asn Gly Ile Glu Glu Asp Ser Asp Glu Pro Leu Glu Arg Arg Leu
 725 730 735
 Ser Leu Val Pro Asp Ser Glu Gln Gly Glu Ala Ile Leu Pro Arg Ile
 740 745 750
 25 Ser Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg Arg Arg Gln Ser
 755 760 765
 Val Leu Asn Leu Met Thr His Ser Val Asn Gln Gly Gln Asn Ile His
 30 770 775 780
 Arg Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu Ala Pro Gln Ala
 785 790 795 800
 35 Asn Leu Thr Glu Leu Asp Ile Tyr Ser Arg Arg Leu Ser Gln Glu Thr
 805 810 815
 Gly Leu Glu Ile Ser Glu Glu Ile Asn Glu Glu Asp Leu Lys Glu Cys
 820 825 830
 40 Leu Phe Asp Asp Met Glu Ser Ile Pro Ala Val Thr Thr Trp Asn Thr
 835 840 845
 Tyr Leu Arg Tyr Ile Thr Val His Lys Ser Leu Ile Phe Val Leu Ile
 45 850 855 860
 Trp Cys Leu Val Ile Phe Leu Ala Glu Val Ala Ala Ser Leu Val Val
 865 870 875 880
 50 Leu Trp Leu Leu Gly Asn Thr Pro Leu Gln Asp Lys Gly Asn Ser Thr
 885 890 895
 His Ser Arg Asn Asn Ser Tyr Ala Val Ile Ile Thr Ser Thr Ser Ser
 900 905 910
 55 Tyr Tyr Val Phe Tyr Ile Tyr Val Gly Val Ala Asp Thr Leu Leu Ala
 915 920 925

Met Gly Phe Phe Arg Gly Leu Pro Leu Val His Thr Leu Ile Thr Val
 930 935 940

5 Ser Lys Ile Leu His His Lys Met Leu His Ser Val Leu Gln Ala Pro
 945 950 955 960

Met Ser Thr Leu Asn Thr Leu Lys Ala Gly Gly Ile Leu Asn Arg Phe
 965 970 975

10 Ser Lys Asp Ile Ala Ile Leu Asp Asp Leu Leu Pro Leu Thr Ile Phe
 980 985 990

Asp Phe Ile Gln Leu Leu Leu Ile Val Ile Gly Ala Ile Ala Val Val
 995 1000 1005

15 Ala Val Leu Gln Pro Tyr Ile Phe Val Ala Thr Val Pro Val Ile Val
 1010 1015 1020

Ala Phe Ile Met Leu Arg Ala Tyr Phe Leu Gln Thr Ser Gln Gln Leu
 1025 1030 1035 1040

Lys Gln Leu Glu Ser Glu Gly Arg Ser Pro Ile Phe Thr His Leu Val
 1045 1050 1055

25 Thr Ser Leu Lys Gly Leu Trp Thr Leu Arg Ala Phe Gly Arg Gln Pro
 1060 1065 1070

Tyr Phe Glu Thr Leu Phe His Lys Ala Leu Asn Leu His Thr Ala Asn
 1075 1080 1085

30 Trp Phe Leu Tyr Leu Ser Thr Leu Arg Trp Phe Gln Met Arg Ile Glu
 1090 1095 1100

Met Ile Phe Val Ile Phe Phe Ile Ala Val Thr Phe Ile Ser Ile Leu
 1105 1110 1115 1120

Thr Thr Gly Glu Gly Glu Gly Arg Val Gly Ile Ile Leu Thr Leu Ala
 1125 1130 1135

40 Met Asn Ile Met Ser Thr Leu Gln Trp Ala Val Asn Ser Ser Ile Asp
 1140 1145 1150

Val Asp Ser Leu Met Arg Ser Val Ser Arg Val Phe Lys Phe Ile Asp
 1155 1160 1165

45 Met Pro Thr Glu Gly Lys Pro Thr Lys Ser Thr Lys Pro Tyr Lys Asn
 1170 1175 1180

Gly Gln Leu Ser Lys Val Met Ile Ile Glu Asn Ser His Val Lys Lys
 1185 1190 1195 1200

Asp Asp Ile Trp Pro Ser Gly Gly Gln Met Thr Val Lys Asp Leu Thr
 1205 1210 1215

55 Ala Lys Tyr Thr Glu Gly Gly Asn Ala Ile Leu Glu Asn Ile Ser Phe
 1220 1225 1230

Ser Ile Ser Pro Gly Gln Arg Val Gly Leu Leu Gly Arg Thr Gly Ser
 1235 1240 1245

5 Gly Lys Ser Thr Leu Leu Ser Ala Phe Leu Arg Leu Leu Asn Thr Glu
 1250 1255 1260

Gly Glu Ile Gln Ile Asp Gly Val Ser Trp Asp Ser Ile Thr Leu Gln
 1265 1270 1275 1280

10 Gln Trp Arg Lys Ala Phe Gly Val Ile Pro Gln Lys Val Phe Ile Phe
 1285 1290 1295

Ser Gly Thr Phe Arg Lys Asn Leu Asp Pro Tyr Glu Gln Trp Ser Asp
 1300 1305 1310

15 Gln Glu Ile Trp Lys Val Ala Asp Glu Val Gly Leu Arg Ser Val Ile
 1315 1320 1325

Glu Gln Phe Pro Gly Lys Leu Asp Phe Val Leu Val Asp Gly Gly Cys
 1330 1335 1340

20 Val Leu Ser His Gly His Lys Gln Leu Met Cys Leu Ala Arg Ser Val
 1345 1350 1355 1360

25 Leu Ser Lys Ala Lys Ile Leu Leu Leu Asp Glu Pro Ser Ala His Leu
 1365 1370 1375

Asp Pro Val Thr Tyr Gln Ile Ile Arg Arg Thr Leu Lys Gln Ala Phe
 1380 1385 1390

30 Ala Asp Cys Thr Val Ile Leu Cys Glu His Arg Ile Glu Ala Met Leu
 1395 1400 1405

Glu Cys Gln Gln Phe Leu Val Ile Glu Glu Asn Lys Val Arg Gln Tyr
 1410 1415 1420

35 Asp Ser Ile Gln Lys Leu Leu Asn Glu Arg Ser Leu Phe Arg Gln Ala
 1425 1430 1435 1440

40 Ile Ser Pro Ser Asp Arg Val Lys Leu Phe Pro His Arg Asn Ser Ser
 1445 1450 1455

Lys Cys Lys Ser Lys Pro Gln Ile Ala Ala Leu Lys Glu Glu Thr Glu
 1460 1465 1470

45 Glu Glu Val Gln Asp Thr Arg Leu
 1475 1480

50 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5635 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

| | | |
|----|-------------------------------------------------------------------|------|
| 5 | CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT | 60 |
| | TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT | 120 |
| | GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGCCGGATG TGGTAAAAGT GACGTTTTTG | 180 |
| 10 | GTGTGCGCCG GTGTATACGG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG | 240 |
| | TAAATTTGGG CGTAACCAAG TAATGTTTGG CCATTTTCGC GGGAAAAGT AATAAGAGGA | 300 |
| 15 | AGTGAAATCT GAATAATTCT GTGTTACTCA TAGCGCGTAA TATTGTCTA GGGCCGCGGG | 360 |
| | GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC | 420 |
| | CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGCTGACG CGCAGTGTAT TTATACCCGG | 480 |
| 20 | TGAGTTCCTC AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC | 540 |
| | TCCGAGCTAG TAACGGCCGC CAGTGTGCTG CAGATATCAA AGTCGACGGT ACCCGAGAGA | 600 |
| 25 | CCATGCAGAG GTCGCCTCTG GAAAAGGCCA GCGTTGTCTC CAAACTTTTT TTCAGCTGGA | 660 |
| | CCAGACCAAT TTTGAGGAAA GGATACAGAC AGCGCCTGGA ATTGTCAGAC ATATACCAAA | 720 |
| | TCCCTTCTGT TGATTCTGCT GACAATCTAT CTGAAAAATT GGAAAGAGAA TGGGATAGAG | 780 |
| 30 | AGCTGGCTTC AAAGAAAAAT CCTAACTCA TTAATGCCCT TCGGCGATGT TTTTCTGGA | 840 |
| | GATTTATGTT CTATGGAATC TTTTATATT TAGGGGAAGT CACCAAAGCA GTACAGCCTC | 900 |
| 35 | TCTTACTGGG AAGAATCATA GCTTCCTATG ACCCGGATAA CAAGGAGGAA CGCTCTATCG | 960 |
| | CGATTTATCT AGGCATAGGC TTATGCCTTC TCTTTATTGT GAGGACACTG CTCCTACACC | 1020 |
| | CAGCCATTTT TGGCCTTCAT CACATTGGAA TGCAGATGAG AATAGCTATG TTTAGTTTGA | 1080 |
| 40 | TTTATAAGAA GACTTTAAAG CTGTCAAGCC GTGTTCTAGA TAAAATAAGT ATTGGACAAC | 1140 |
| | TTGTTAGTCT CCTTTCCAAC AACCTGAACA AATTGATGA AGGACTTGCA TTGGCACATT | 1200 |
| 45 | TCGTGTGGAT CGCTCCTTTG CAAGTGGCAC TCCTCATGGG GCTAATCTGG GAGTTGTTAC | 1260 |
| | AGGCGTCTGC CTTCTGTGGA CTTGGTTTCC TGATAGTCCT TGCCCTTTTT CAGGCTGGGC | 1320 |
| | TAGGGAGAAT GATGATGAAG TACAGAGATC AGAGAGCTGG GAAGATCAGT GAAAGACTTG | 1380 |
| 50 | TGATTACCTC AGAAATGATT GAAAACATCC AATCTGTTAA GGCATACTGC TGGGAAGAAG | 1440 |
| | CAATGGAAAA AATGATTGAA AACTTAAGAC AAACAGAACT GAAACTGACT CGGAAGGCAG | 1500 |
| 55 | CCTATGTGAG ATACTTCAAT AGCTCAGCCT TCTTCTTCTC AGGGTTCTTT GTGGTGTTTT | 1560 |
| | TATCTGTGCT TCCCTATGCA CTAATCAAAG GAATCATCCT CCGGAAAATA TTCACCACCA | 1620 |
| | TCTCATTCTG CATTGTTCTG CGCATGGCGG TCACTCGGCA ATTTCCCTGG GCTGTACAAA | 1680 |

| | | | | | | | |
|----|------------|------------|------------|------------|------------|-------------|------|
| | CATGGTATGA | CTCTCTTGGA | GCAATAAACA | AAATACAGGA | TTTCTTACAA | AAGCAAGAAT | 1740 |
| | ATAAGACATT | GGAATATAAC | TTAACGACTA | CAGAAGTAGT | GATGGAGAAT | GTAACAGCCT | 1800 |
| 5 | TCTGGGAGGA | GGGATTTGGG | GAATTATTTG | AGAAAGCAAA | ACAAAACAAT | AACAATAGAA | 1860 |
| | AAACTTCTAA | TGGTGATGAC | AGCCTCTTCT | TCAGTAATTT | CTCACTTCTT | GGTACTCCTG | 1920 |
| | TCCTGAAAGA | TATTAATTTT | AAGATAGAAA | GAGGACAGTT | GTTGGCGGTT | GCTGGATCCA | 1980 |
| 10 | CTGGAGCAGG | CAAGACTTCA | CTTCTAATGA | TGATTATGGG | AGAACTGGAG | CCTTCAGAGG | 2040 |
| | GTAAAATTAA | GCACAGTGGA | AGAATTTTCT | TCTGTTCTCA | GTTTTCTCTG | ATTATGCCTG | 2100 |
| 15 | GCACCATTAA | AGAAAATATC | ATCTTTGGTG | TTTCCTATGA | TGAATATAGA | TACAGAAGCG | 2160 |
| | TCATCAAAGC | ATGCCAACTA | GAAGAGGACA | TCTCCAAGTT | TGCAGAGAAA | GACAATATAG | 2220 |
| | TTCTTGGAGA | AGGTGGAATC | ACACTGAGTG | GAGGTCAACG | AGCAAGAATT | TCTTTAGCAA | 2280 |
| 20 | GAGCAGTATA | CAAAGATGCT | GATTTGTATT | TATTAGACTC | TCCTTTTGGG | TACCTAGATG | 2340 |
| | TTTAAACAGA | AAAAGAAATA | TTTGAAAGCT | GTGTCTGTAA | ACTGATGGCT | AACAAAATA | 2400 |
| 25 | GGATTTTGGT | CACTTCTAAA | ATGGAACATT | TAAAGAAAGC | TGACAAAATA | TTAATTTTGC | 2460 |
| | ATGAAGGTAG | CAGCTATTTT | TATGGGACAT | TTTCAGAACT | CCAAAATCTA | CAGCCAGACT | 2520 |
| | TTAGCTCAAA | ACTCATGGGA | TGTGATTCTT | TCGACCAATT | TAGTGCAGAA | AGAAGAAATT | 2580 |
| 30 | CAATCCTAAC | TGAGACCTTA | CACCGTTTCT | CATTAGAAGG | AGATGCTCCT | GTCTCCTGGA | 2640 |
| | CAGAAACAAA | AAAACAATCT | TTTAAACAGA | CTGGAGAGTT | TGGGGAAAAA | AGGAAGAATT | 2700 |
| 35 | CTATTCTCAA | TCCAATCAAC | TCTATACGAA | AATTTTCCAT | TGTGCAAAAG | ACTCCCTTAC | 2760 |
| | AAATGAATGG | CATCGAAGAG | GATTCTGATG | AGCCTTTAGA | GAGAAGGCTG | TCCTTAGTAC | 2820 |
| | CAGATTCTGA | GCAGGGAGAG | GCGATACTGC | CTCGCATCAG | CGTGATCAGC | ACTGGCCCCA | 2880 |
| 40 | CGCTTCAGGC | ACGAAGGAGG | CAGTCTGTCC | TGAACCTGAT | GACACACTCA | GTTAACCAAG | 2940 |
| | GTCAGAACAT | TCACCGAAAG | ACAACAGCAT | CCACACGAAA | AGTGTCCTG | GCCCCCTCAGG | 3000 |
| 45 | CAAACTTGAC | TGAACTGGAT | ATATATTCAA | GAAGGTTATC | TCAAGAAACT | GGCTTGAAAA | 3060 |
| | TAAGTGAAGA | AATTAACGAA | GAAGACTTAA | AGGAGTGCCT | TTTTGATGAT | ATGGAGAGCA | 3120 |
| | TACCAGCAGT | GACTACATGG | AACACATACC | TTGATATAT | TACTGTCCAC | AAGAGCTTAA | 3180 |
| 50 | TTTTTGTGCT | AATTTGGTGC | TTAGTAATTT | TTCTGGCAGA | GGTGGCTGCT | TCTTTGGTTG | 3240 |
| | TGCTGTGGCT | CCTTGGAAC | ACTCCTCTTC | AAGACAAAGG | GAATAGTACT | CATAGTAGAA | 3300 |
| 55 | ATAACAGCTA | TGCAGTGATT | ATCACCAGCA | CCAGTTCGTA | TTATGTGTTT | TACATTTACG | 3360 |
| | TGGGAGTAGC | CGACACTTTG | CTTGCTATGG | GATTCCTCAG | AGGTCTACCA | CTGGTGCATA | 3420 |
| | CTCTAATCAC | AGTGTCGAAA | ATTTTACACC | ACAAAATGTT | ACATTCTGTT | CTTCAAGCAC | 3480 |

| | | |
|----|--------------------------------------------------------------------|------|
| | CTATGTCAAC CCTCAACACG TTGAAAGCAG GTGGGATTCT TAATAGATTC TCCAAAGATA | 3540 |
| 5 | TAGCAATTTT GGATGACCTT CTGCCTCTTA CCATATTTGA CTTTCATCCAG TTGTTATTAA | 3600 |
| | TTGTGATTGG AGCTATAGCA GTTGTGCGAG TTTTACAACC CTACATCTTT GTTGCAACAG | 3660 |
| | TGCCAGTGAT AGTGGCTTTT ATTATGTTGA GAGCATATTT CCTCCAAACC TCACAGCAAC | 3720 |
| 10 | TCAAACAAC TGAATCTGAA GGCAGGAGTC CAATTTTCAC TCATCTTGTT ACAAGCTTAA | 3780 |
| | AAGGACTATG GACACTTCGT GCCTTCGGAC GGCAGCCTTA CTTTGAAACT CTGTTCCACA | 3840 |
| 15 | AAGCTCTGAA TTTACATACT GCCAACTGGT TCTTGACCT GTCAACACTG CGCTGGTTCC | 3900 |
| | AAATGAGAAT AGAAATGATT TTTGTCTCT TCTTCATTGC TGTTACCTTC ATTTCCATTT | 3960 |
| | TAACAACAGG AGAAGGAGAA GGAAGAGTTG GTATTATCCT GACTTTAGCC ATGAATATCA | 4020 |
| 20 | TGAGTACATT GCAGTGGGCT GTAAACTCCA GCATAGATGT GGATAGCTTG ATGCGATCTG | 4080 |
| | TGAGCCGAGT CTTTAAGTTC ATTGACATGC CAACAGAAGG TAAACCTACC AAGTCAACCA | 4140 |
| 25 | AACCATACAA GAATGGCCAA CTCTCGAAAG TTATGATTAT TGAGAATTCA CACGTGAAGA | 4200 |
| | AAGATGACAT CTGGCCCTCA GGGGGCCAAA TGACTGTCAA AGATCTCACA GCAAAATACA | 4260 |
| | CAGAAGGTGG AAATGCCATA TTAGAGAACA TTCCTTCTC AATAAGTCCT GGCCAGAGGG | 4320 |
| 30 | TGGGCCTCTT GGAAGAAGT GGATCAGGGA AGAGTACTTT GTTATCAGCT TTTTGTAGAC | 4380 |
| | TACTGAACAC TGAAGGAGAA ATCCAGATCG ATGGTGTGTC TTGGGATTCA ATAACTTTGC | 4440 |
| 35 | AACAGTGGAG GAAAGCCTTT GGAGTGATAC CACAGAAAGT ATTTATTTTT TCTGGAACAT | 4500 |
| | TTAGAAAAA CTTGGATCCC TATGAACAGT GGAGTGATCA AGAAATATGG AAAGTTGCAG | 4560 |
| | ATGAGGTTGG GCTCAGATCT GTGATAGAAC AGTTTCCTGG GAAGCTTGAC TTTGTCCTTG | 4620 |
| 40 | TGGATGGGG CTGTGTCCTA AGCCATGGCC ACAAGCAGTT GATGTGCTTG GCTAGATCTG | 4680 |
| | TTCTCAGTAA GCGAAGATC TTGCTGCTTG ATGAACCCAG TGCTCATTTG GATCCAGTAA | 4740 |
| 45 | CATACCAAAT AATTAGAAGA ACTCTAAAAC AAGCATTTGC TGATTGCACA GTAATTCTCT | 4800 |
| | GTGAACACAG GATAGAAGCA ATGCTGGAAT GCCAACAATT TTTGGTCATA GAAGAGAACA | 4860 |
| | AAGTGCGGCA GTACGATTCC ATCCAGAAAC TGCTGAACGA GAGGAGCCTC TTCCGGCAAG | 4920 |
| 50 | CCATCAGCCC CTCCGACAGG GTGAAGCTCT TTCCCCACCG GAACTCAAGC AAGTGCAAGT | 4980 |
| | CTAAGCCCCA GATTGCTGCT CTGAAAGAGG AGACAGAAGA AGAGGTGCAA GATACAAGGC | 5040 |
| 55 | TTTAGAGAGC AGCATAAATG TTGACATGGG ACATTTGCTC ATGGAATTGG AGGTAGCGGA | 5100 |
| | TTGAGGTACT GAAATGTGTG GCGTGGCTT AAGGGTGGGA AAGAATATAT AAGGTGGGGG | 5160 |
| | TCTCATGTAG TTTTGTATCT GTTTTGCAGC AGCCGCCGCC ATGAGCGCCA ACTCGTTTGA | 5220 |

2145641

- 113 -

TGGAAGCATT GTGAGCTCAT ATTTGACAAC GCGCATGCCC CCATGGGCCG GGGTGCCTCA 5280
GAATGTGATG GGCTCCAGCA TTGATGGTCG CCCCCTCCTG CCCGCAAACCT CTACTACCTT 5340
5 GACCTACGAG ACCGTGTCTG GAACGCCGTT GGAGACTGCA GCCTCCGCCG CCGCTTCAGC 5400
CGCTGCAGCC ACCGCCCCGCG GGATTGTGAC TGACTTTGCT TTCCTGAGCC CGCTTGCAAG 5460
CAGTGCAGCT TCCCGTTTCAT CCGCCCCGCGA TGACAAAGTTG ACGGCTCTTT TGGCACAATT 5520
10 GGATTCTTTG ACCCGGGAAC TTAATGTCGT TTCTCAGCAG CTGTTGGATC TGCGCCAGCA 5580
GGTTTCTGCC CTGAAGGCTT CCTCCCCCTCC CAATGCGGTT TAAACATAA ATAAA 5635

15 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTCTTGAGT GCCAGCGAGT AGAGTTTTCT CCTCCG

36

30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

35 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

45 GCAAAGGAGC GATCCACACG AAATGTGCC

29

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

50 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCCTCCGAG CCGCTCCGAG CTAG

24

(2) INFORMATION FOR SEQ ID NO:7:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

10

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCAAAAATGG CTGGGTGTAG GAGCAGTGTC C

31

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid

25

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGATCCTTT ATTATAGGGG AAGTCCACGC CTAC

34

35

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

40

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGGATCCAT CGATGAAATA TGACTACGTC CG

32

50

72648-7

115

CLAIMS:

1. An adenoviral vector comprising an adenovirus genome from which the E1, E2, E3, and E4 regions and late genes of the adenovirus genome have been deleted, a genetic material of interest operably linked to expression control sequences and the 5' and 3' inverted terminal repeat sequences, 5' packaging sequences and the E1A enhancer sequences of the adenovirus genome.
2. The adenoviral vector of claim 1 wherein the adenoviral vector further comprises a nuclear localization signal and a polyadenylation signal.
3. The adenoviral vector of claim 1 or 2 wherein the adenoviral vector control sequences comprise a phosphoglycerate kinase (PGK) promoter.
4. The adenoviral vector of any one of claims 1 to 3 wherein the 5' inverted terminal repeat sequences, the 5' packaging sequences and the E1A enhancer sequence comprise nucleotides 1-358 of the Ad2 adenoviral genome.
5. The adenoviral vector of any one of claims 1 to 3 wherein the 5' inverted terminal repeat sequences, the 5' packaging sequences and the E1A enhancer sequence comprise nucleotides 1-380 of the Ad2 adenoviral genome.
6. The adenoviral vector of claim 2 wherein the expression control sequences comprise the adenovirus major late promoter and the adenovirus tripartite leader, the nuclear localization signal comprises the SV40 T-antigen nuclear localization signal, and the polyadenylation signal comprises the SV40 polyadenylation signal.

72648-7

116

7. The adenoviral vector of claim 6 wherein the adenoviral vector further comprises an internal ribosomal entry site.
8. The adenoviral vector of claim 7 wherein the internal
5 ribosomal entry signal is an internal initiation signal.
9. The adenoviral vector of any one of claims 1 to 8, wherein the genetic material of interest encodes Factor VIII.
10. The adenoviral vector of any one of claims 1 to 8, wherein the genetic material of interest comprises more than
10 one Factor VIII-encoding DNA sequences.
11. The adenoviral vector of any one of claims 1 to 8, wherein the genetic material of interest encodes Factor IX.
12. The adenoviral vector of any one of claims 1 to 8, wherein the genetic material of interest comprises DNA encoding
15 cystic fibrosis transmembrane conductance regulator.
13. The adenoviral vector of any one of claims 1 to 8, wherein the genetic material of interest comprises more than one Factor IX- encoding DNA sequences.
14. A pharmaceutical composition comprising the
20 adenoviral vector according to any one of claims 1 to 13 and a suitable carrier or diluent.
15. A pseudo-adenovirus (PAV)I vector.
16. A pseudo-adenovirus (PAV)II vector.

SMART & BIGGAR
OTTAWA, CANADA

PATENT AGENTS

Application number / numéro de demande: 214 564/

Figures: 16-17-19-20A-20B-21A-21B-21C

~~Pages:~~ 21D-22A-22B-22C-22D-24

25A to 25I-26-36A to 36D

37A to 37D-38A to 38D-42-43

44

Unscannable item(s)

received with this application

To inquire if you can order a copy of the unscannable items, please visit the
CIPO WebSite at [HTTP://CIPO.GC.CA](http://CIPO.GC.CA)

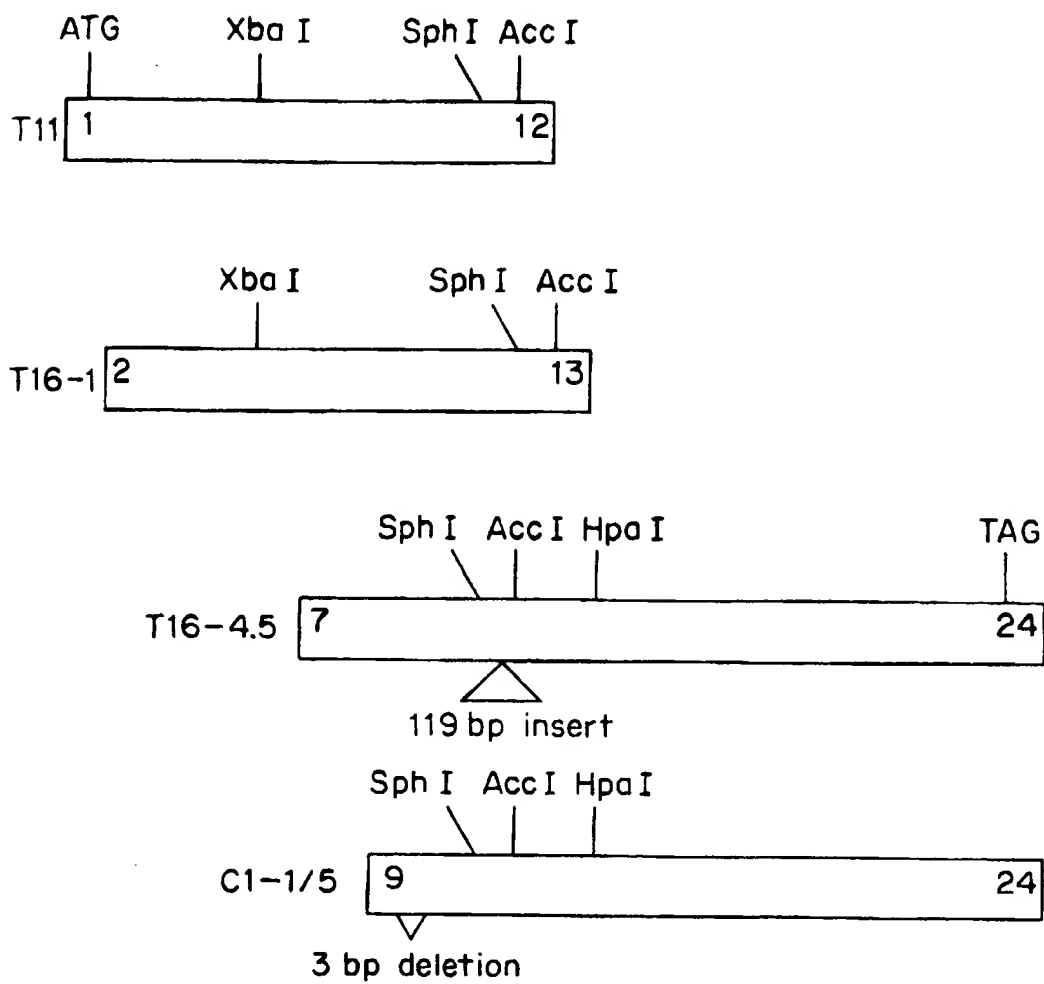
Item(s) ne pouvant être balayés

Documents reçus avec cette demande ne pouvant être balayés.

Pour vous renseigner si vous pouvez commander une copie des items ne
pouvant être balayés, veuillez visiter le site web de l'OPIC au [HTTP://CIPO.C](http://CIPO.C)

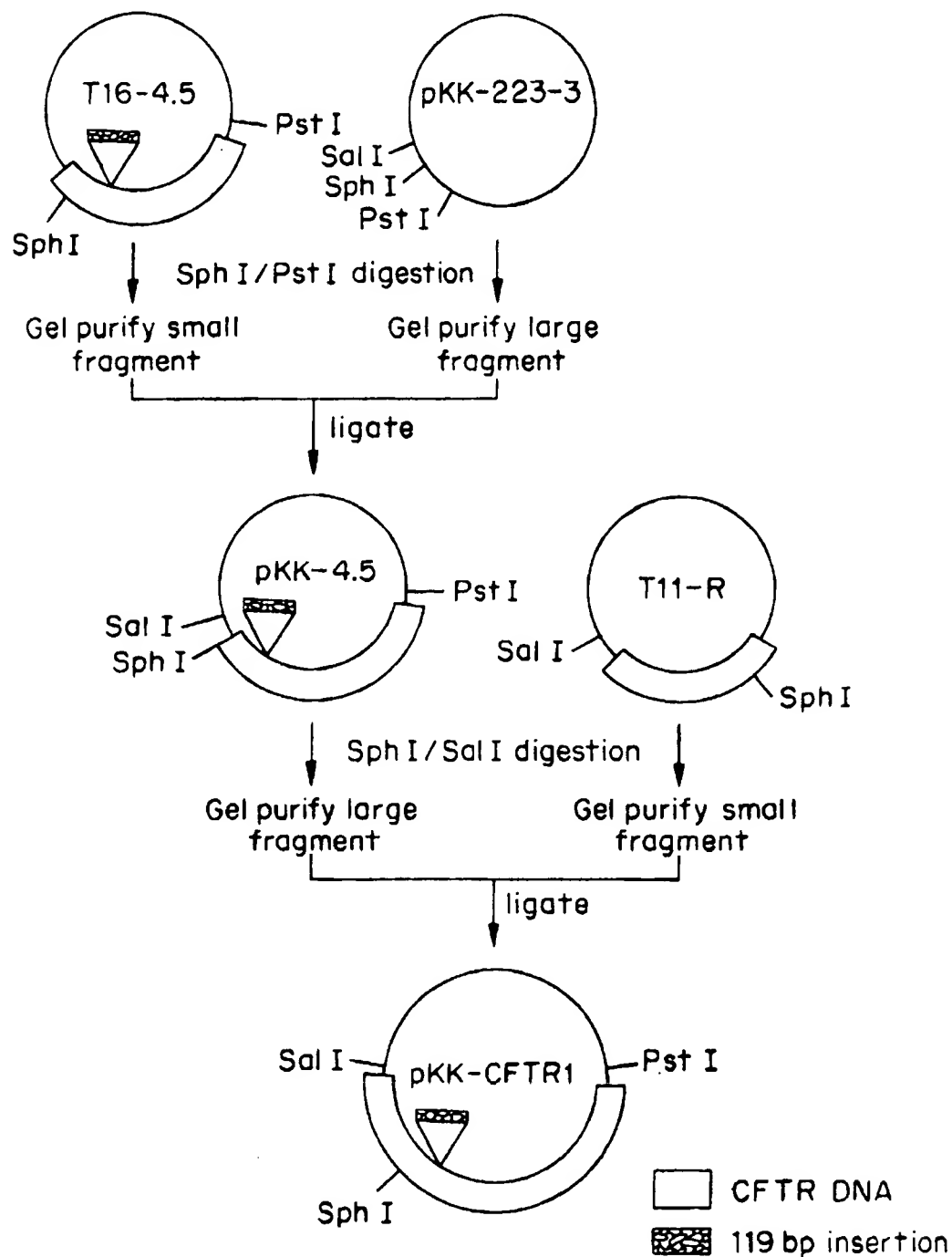
1/56

PARTIAL cDNA CLONES OF THE CFTR GENE

**FIG. 1**

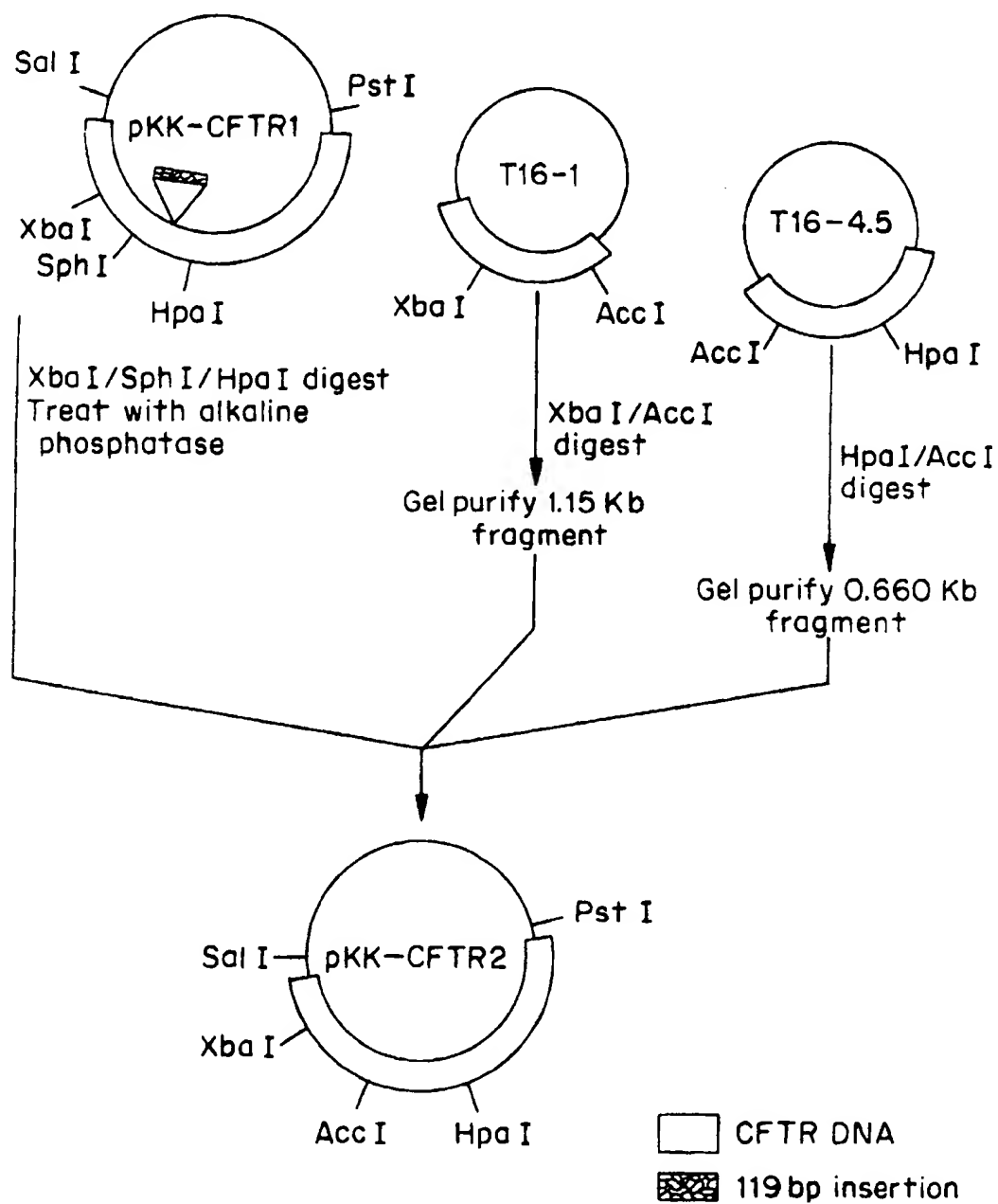
2/56

STRATEGY FOR CONSTRUCTING pKK-CFTR1

**FIG. 2**

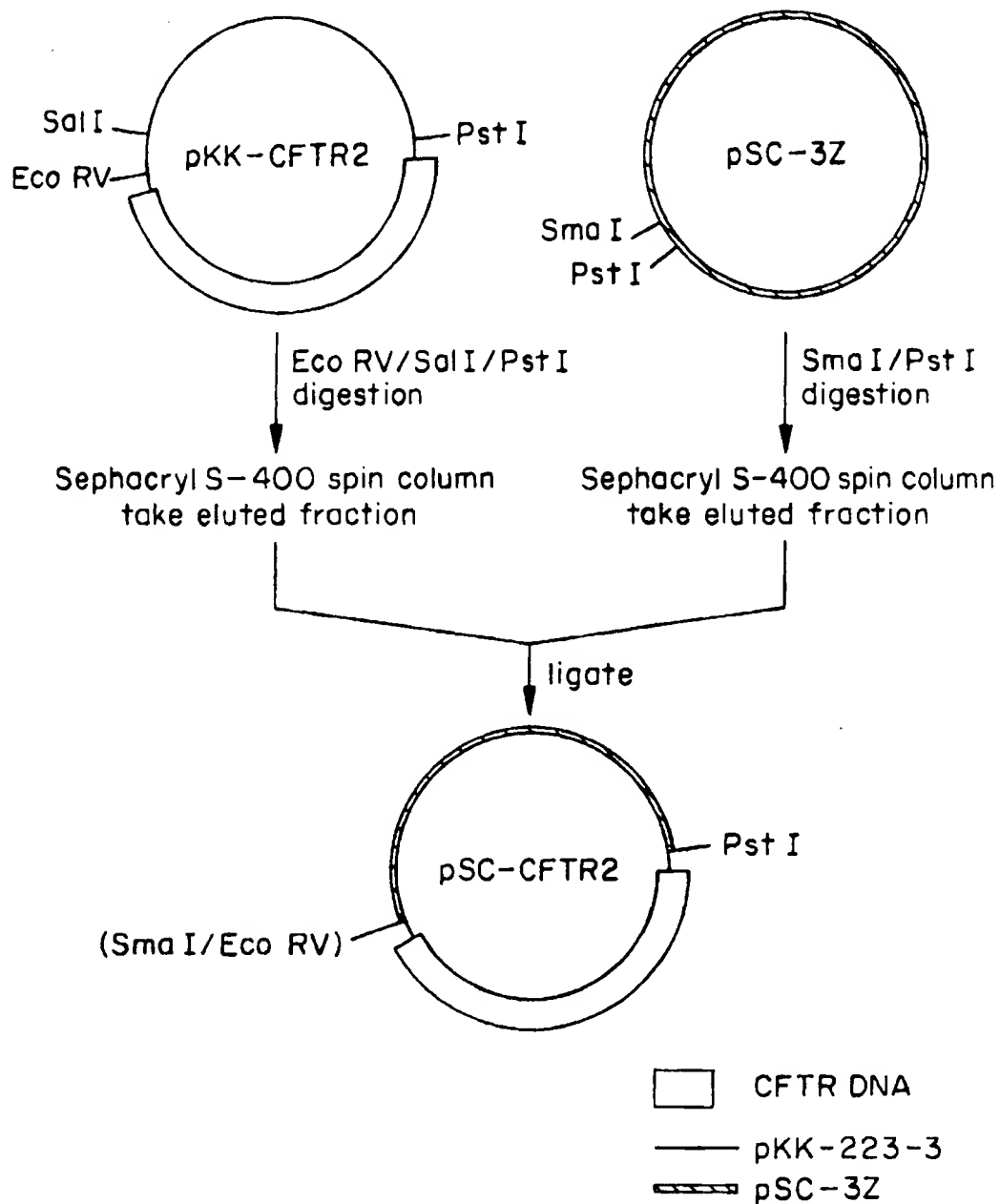
3/56

CONSTRUCTION OF THE pKK-CFTR2 PLASMID

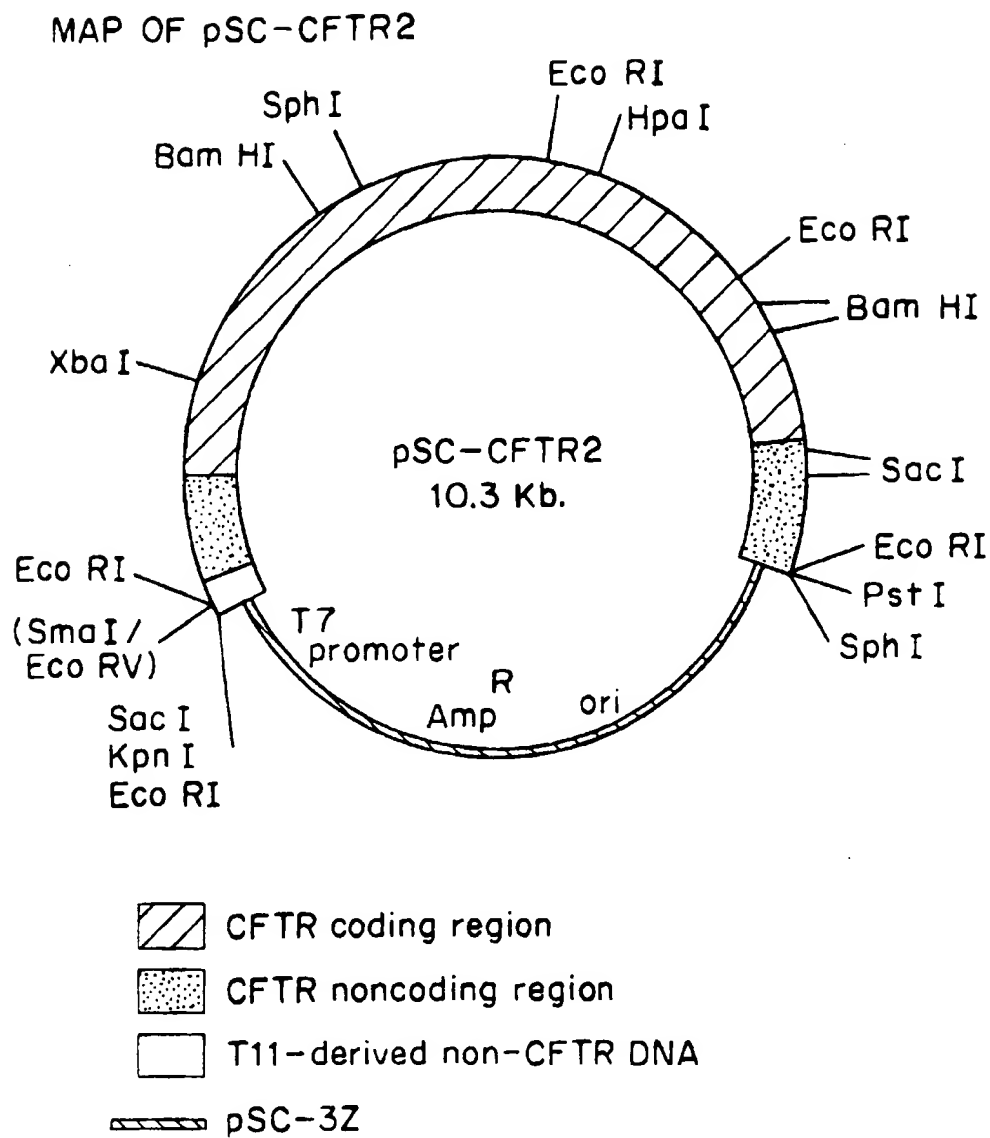
**FIG. 3**

4/56

STRATEGY FOR CONSTRUCTING THE pSC-CFTR2 PLASMID

**FIG. 4**

5/56

**FIG. 5**

6/56

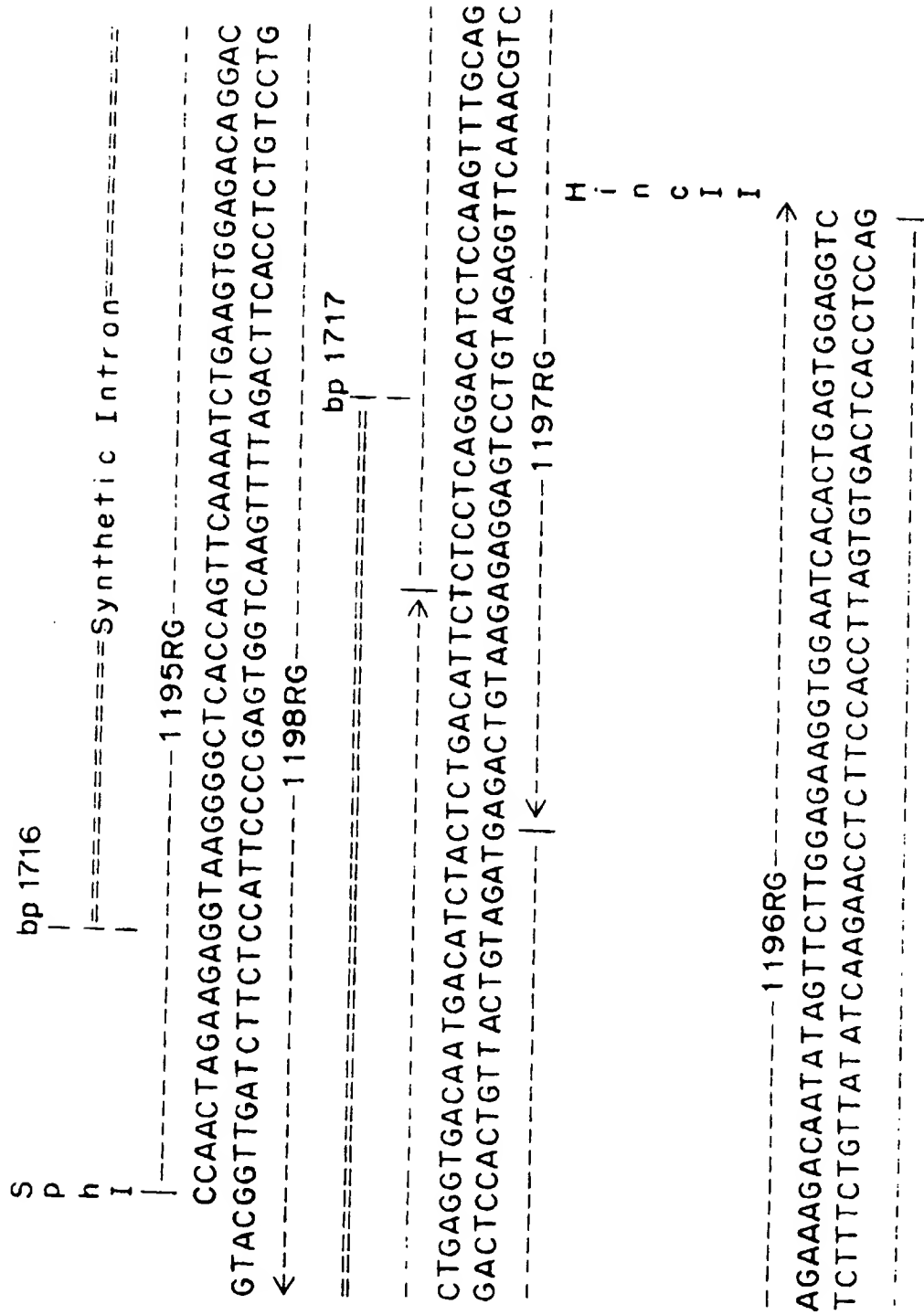


FIG. 6

7/56

CONSTRUCTION OF THE pKK-CFTR3 cDNA

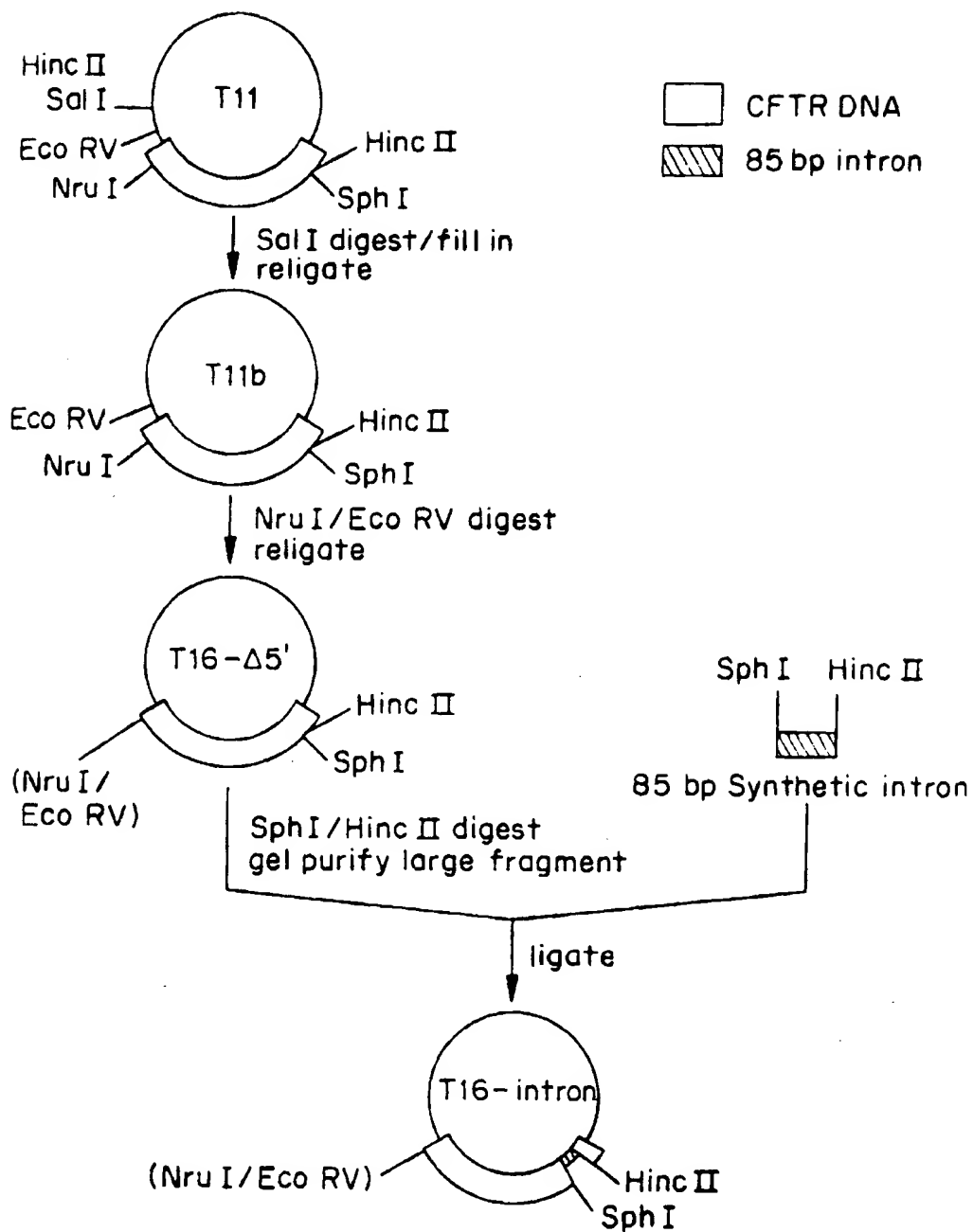
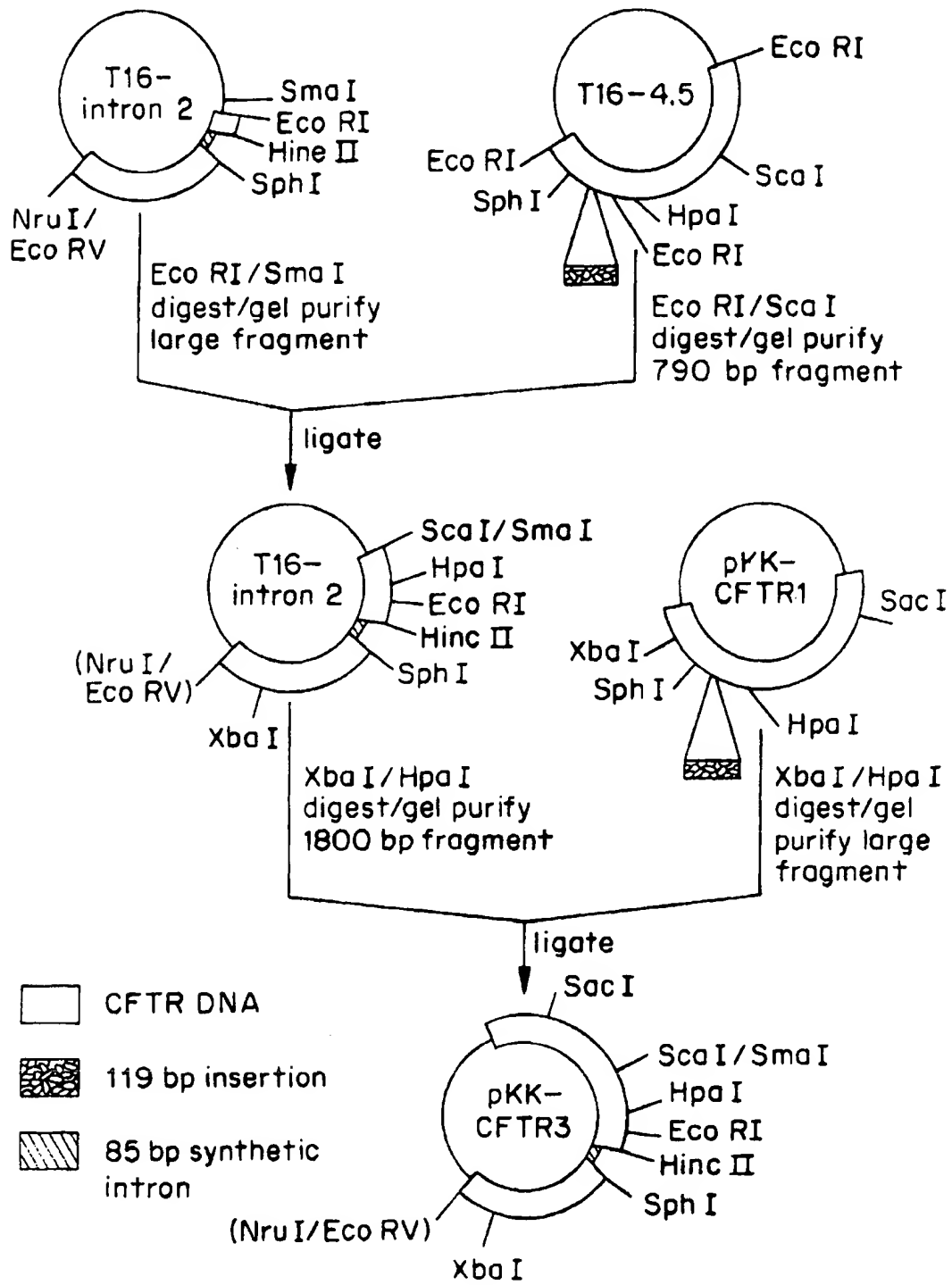


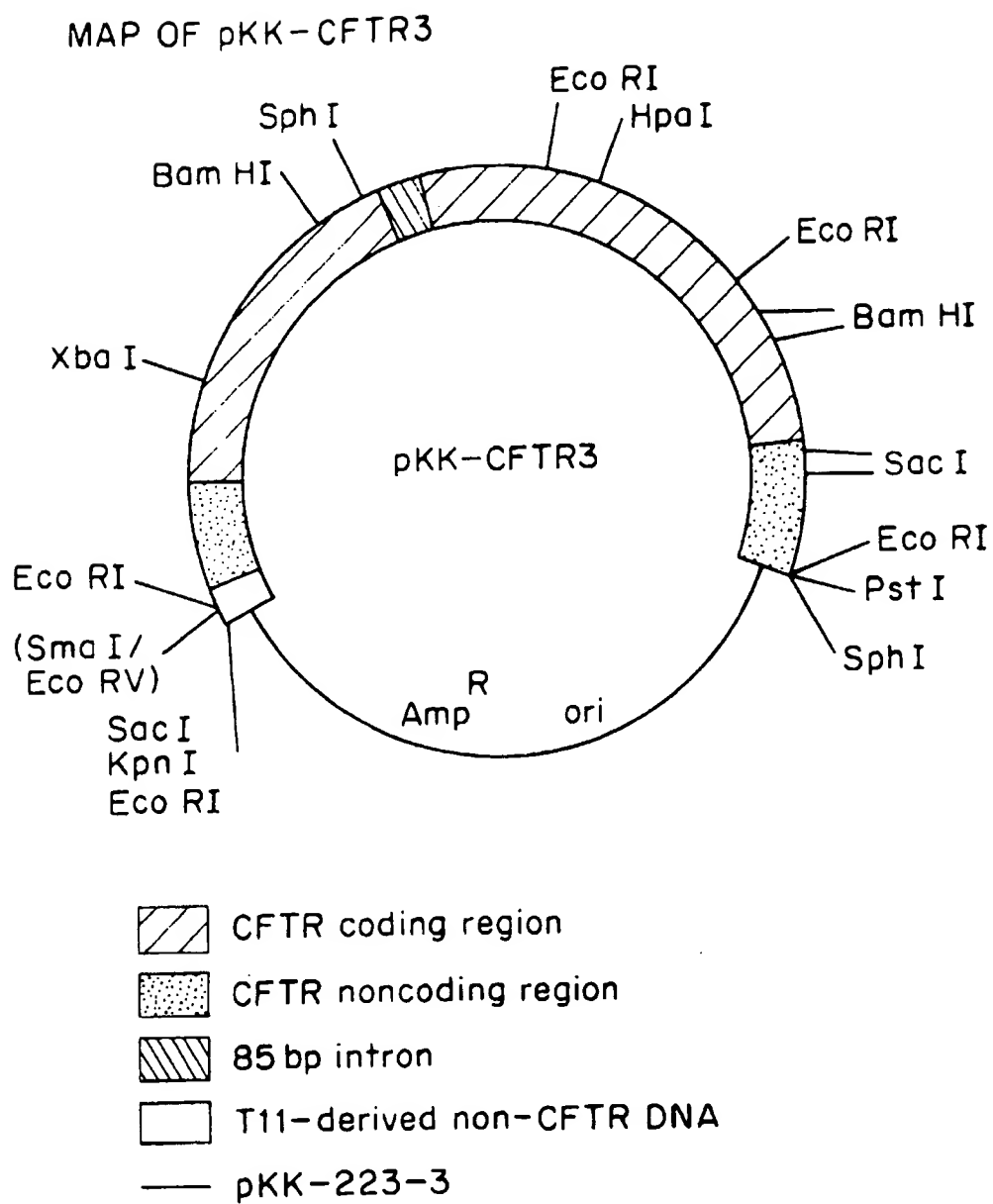
FIG. 7A

8/56

CONSTRUCTION OF THE pKK-CFTR3 CLONE (cont'd)

**FIG. 7B**

9/56

**FIG. 8**

10/56

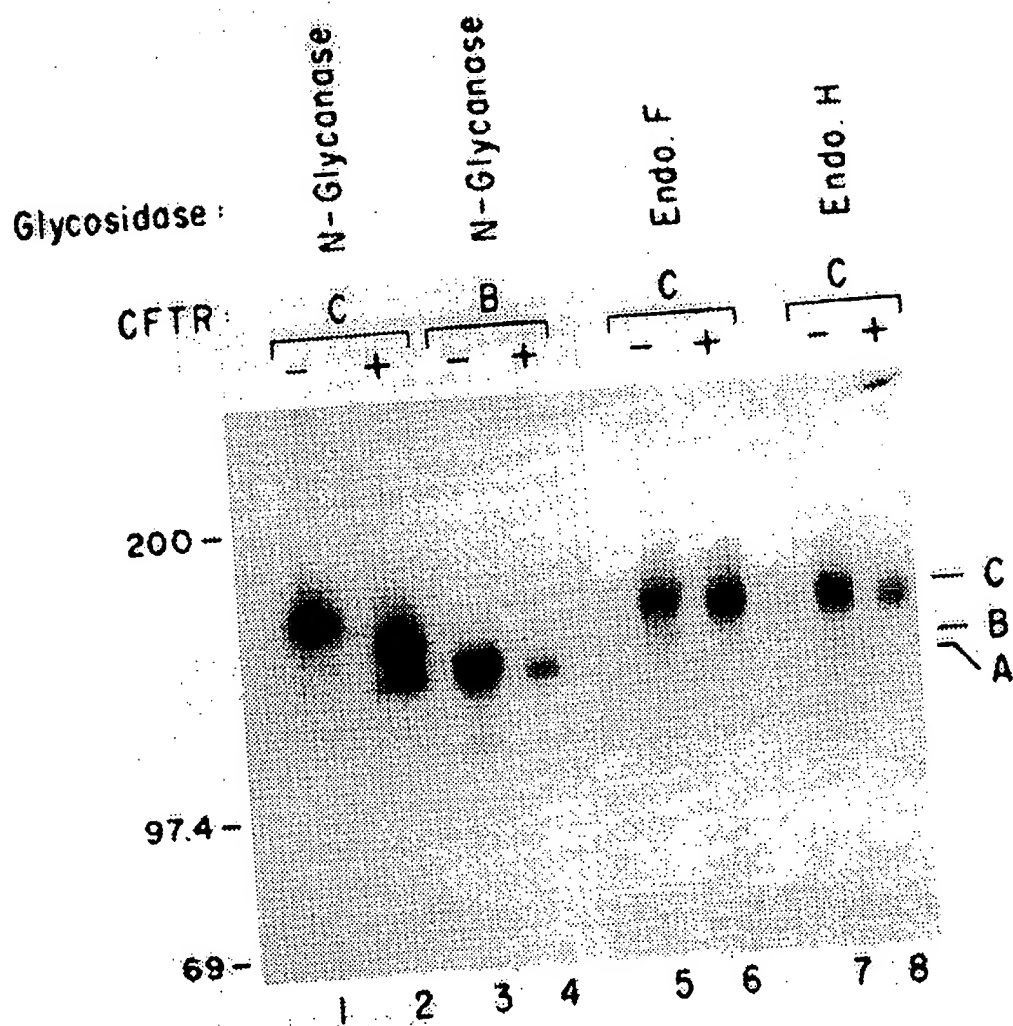


FIG. 9

11/56

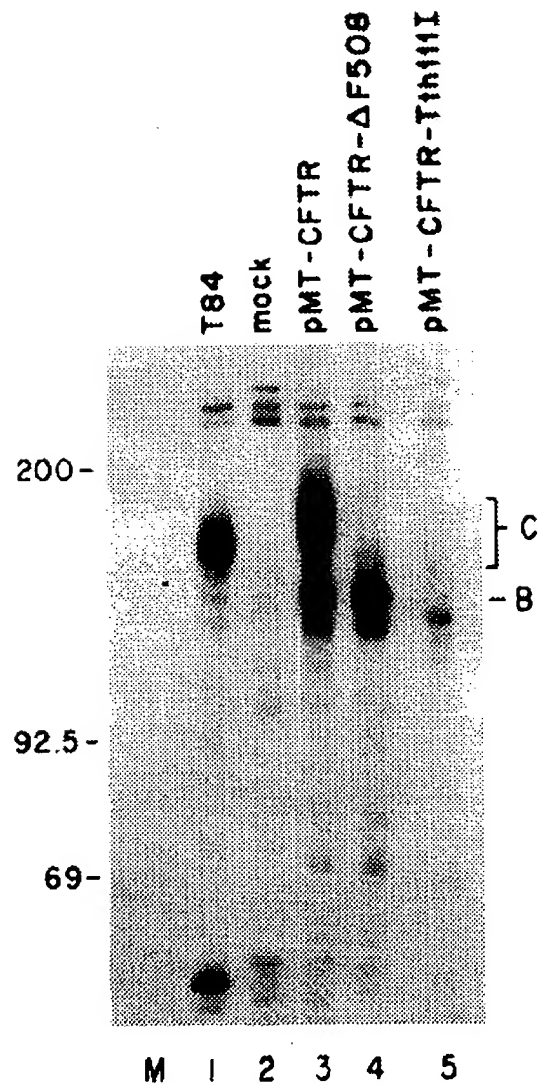
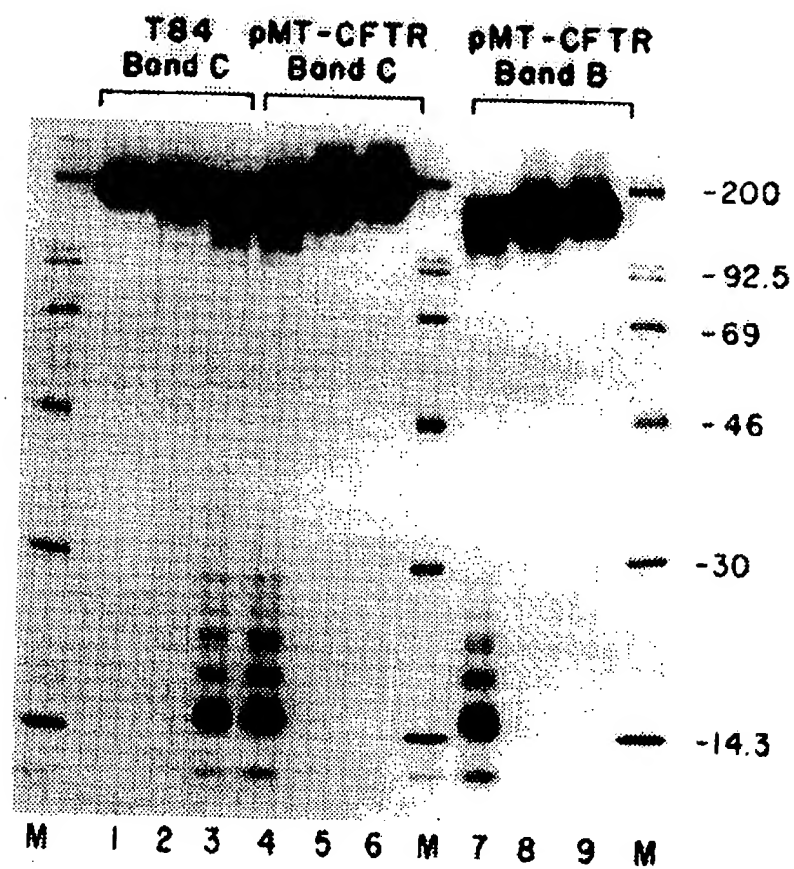
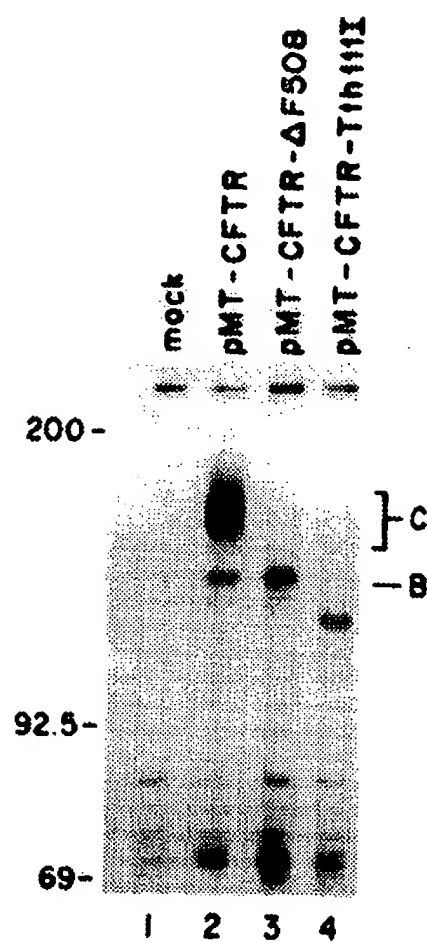


FIG. 10A

12/56

**FIG. 10B**

13/56

**FIG. 11A**

14/56

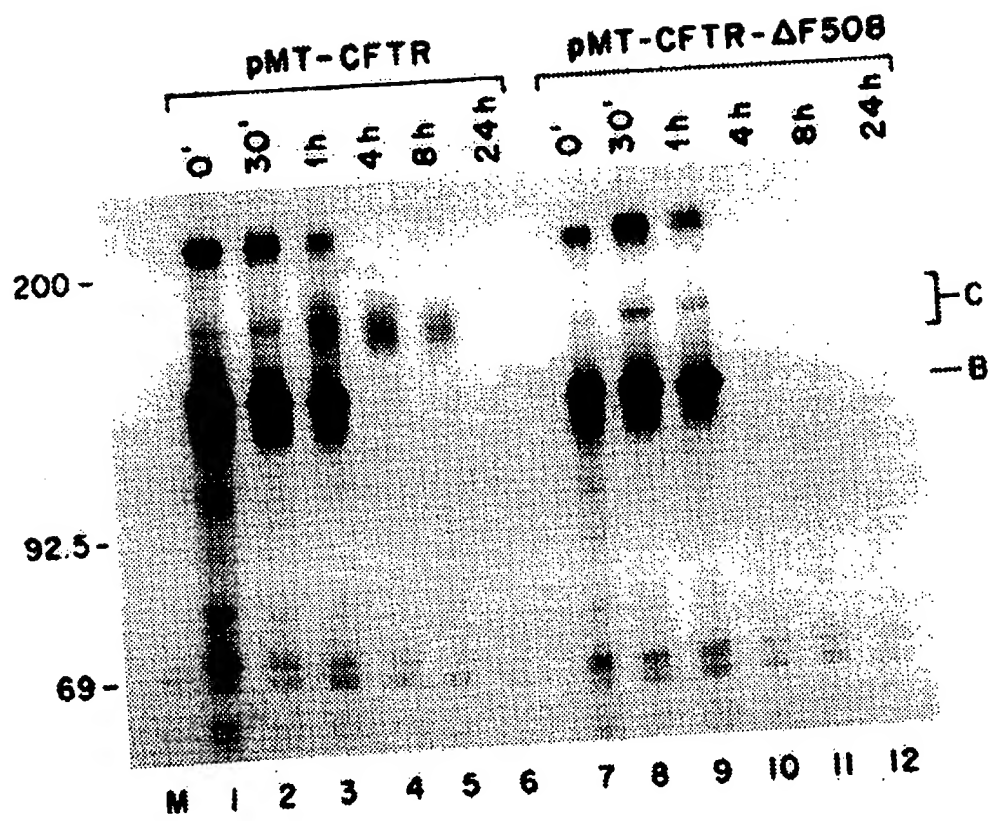


FIG. 11B

15/56

FIG. 12A

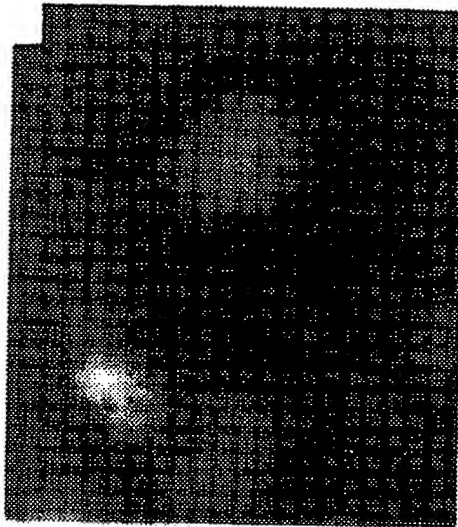


FIG. 12B

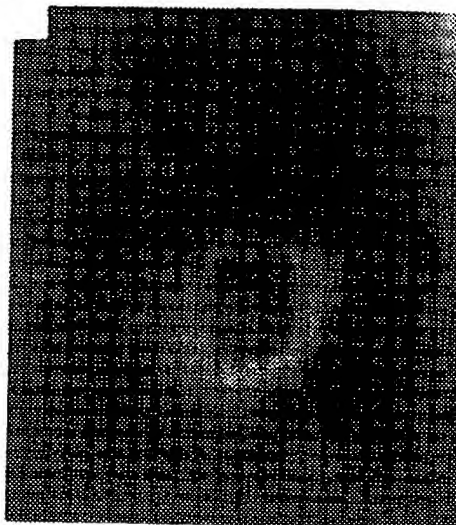
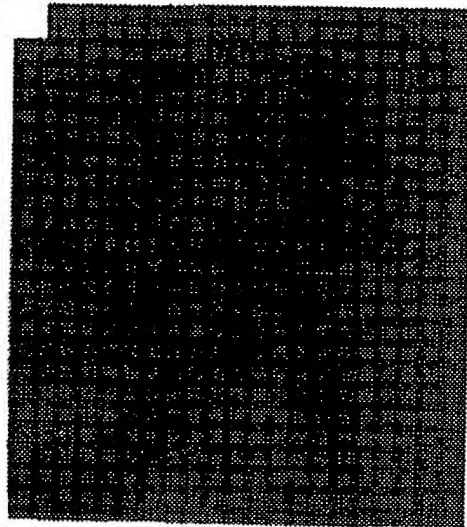


FIG. 12C

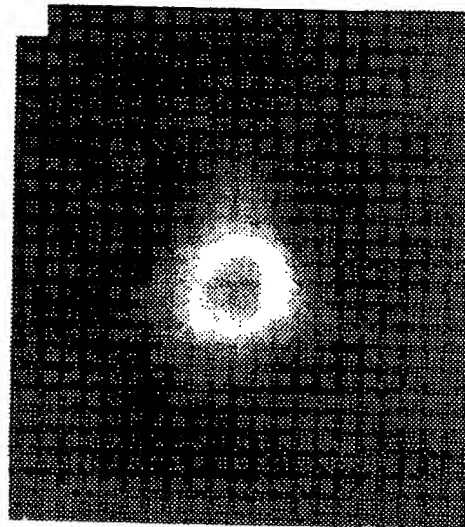


FIG. 12D

16/56

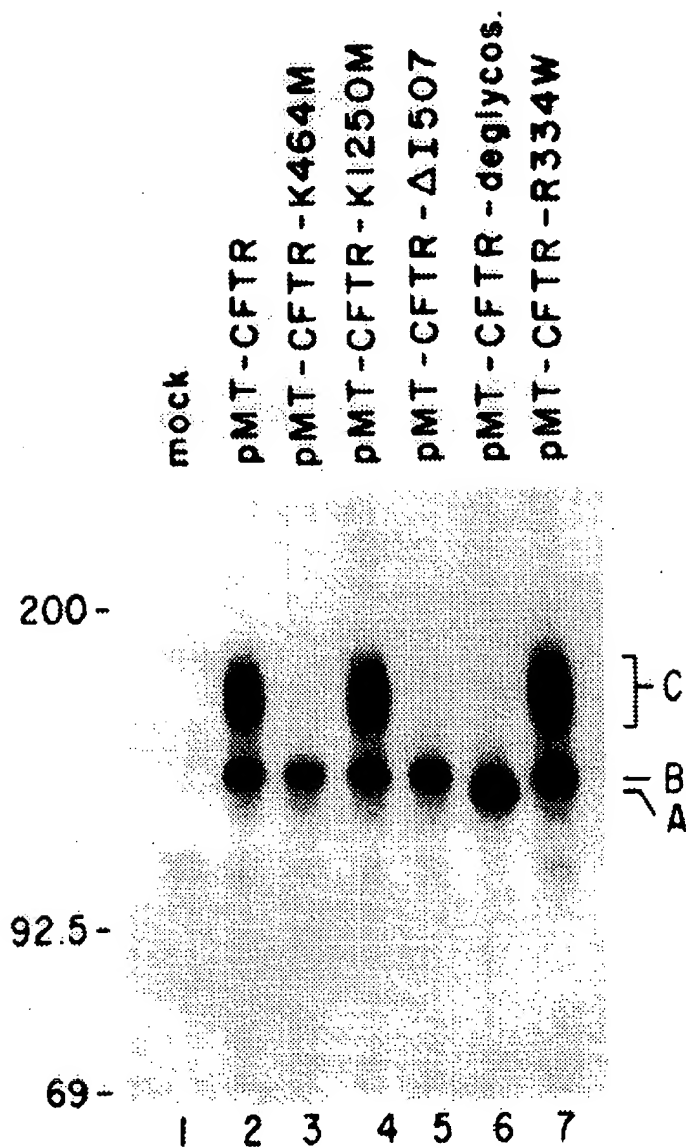


FIG. 13

17/56

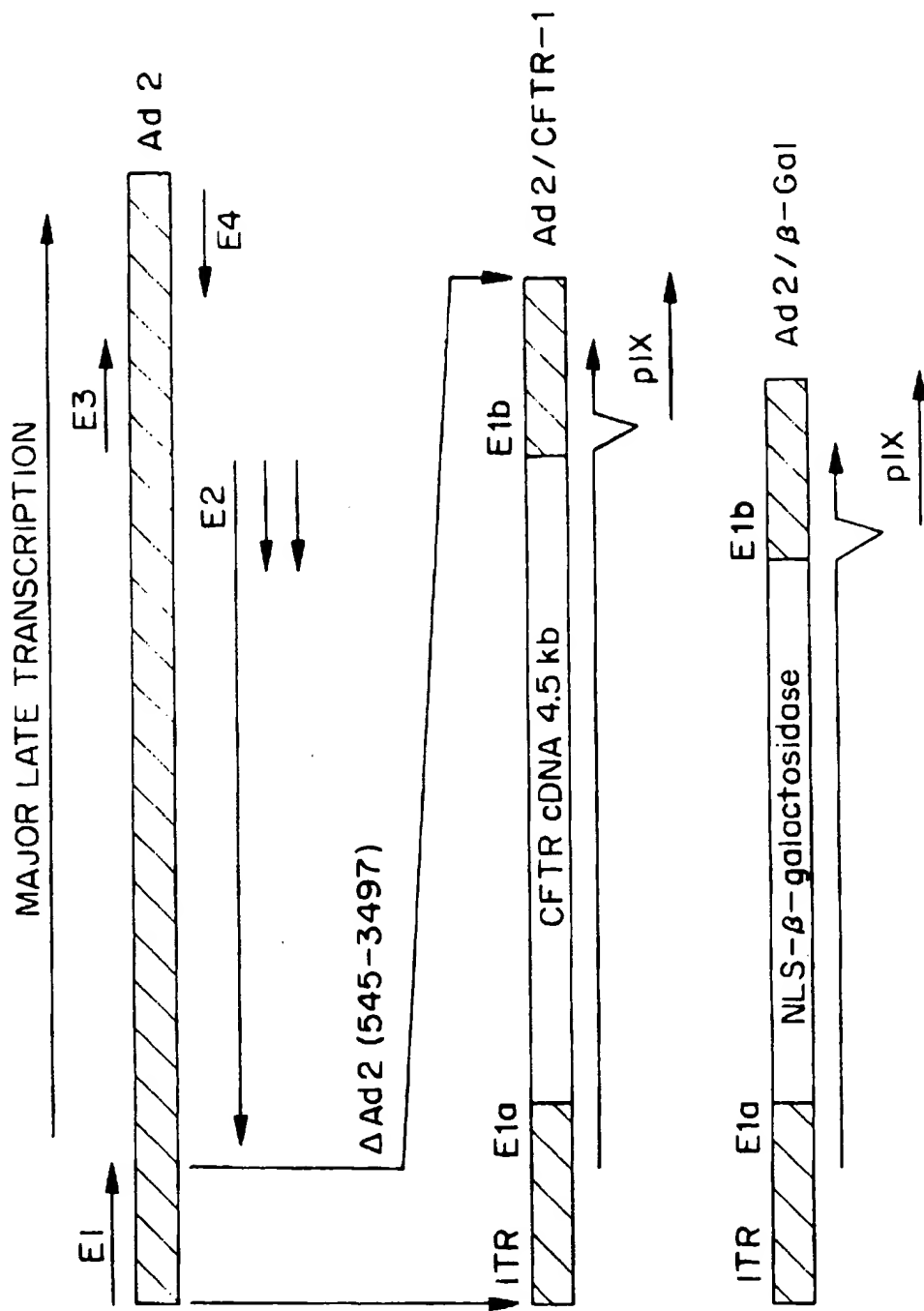
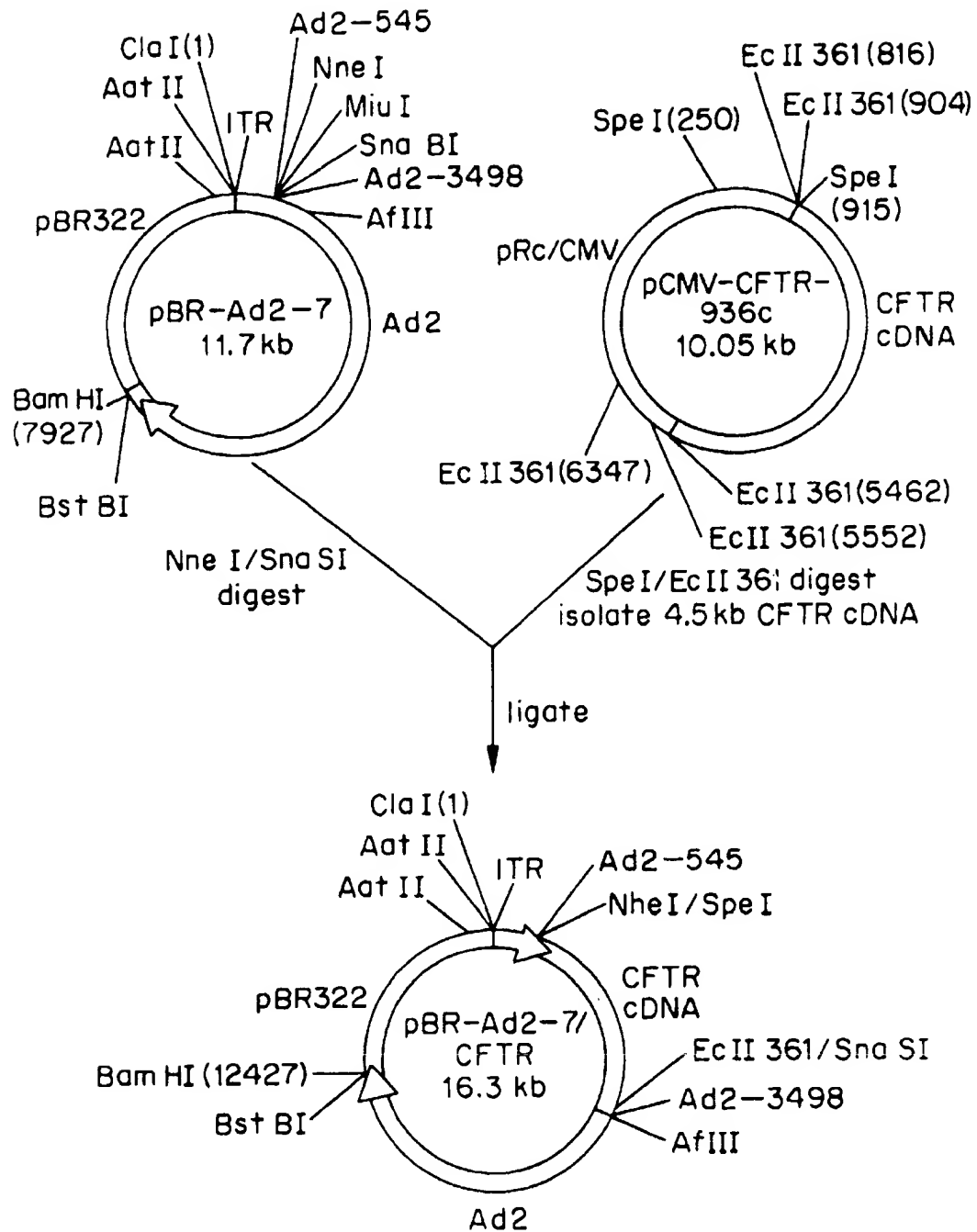
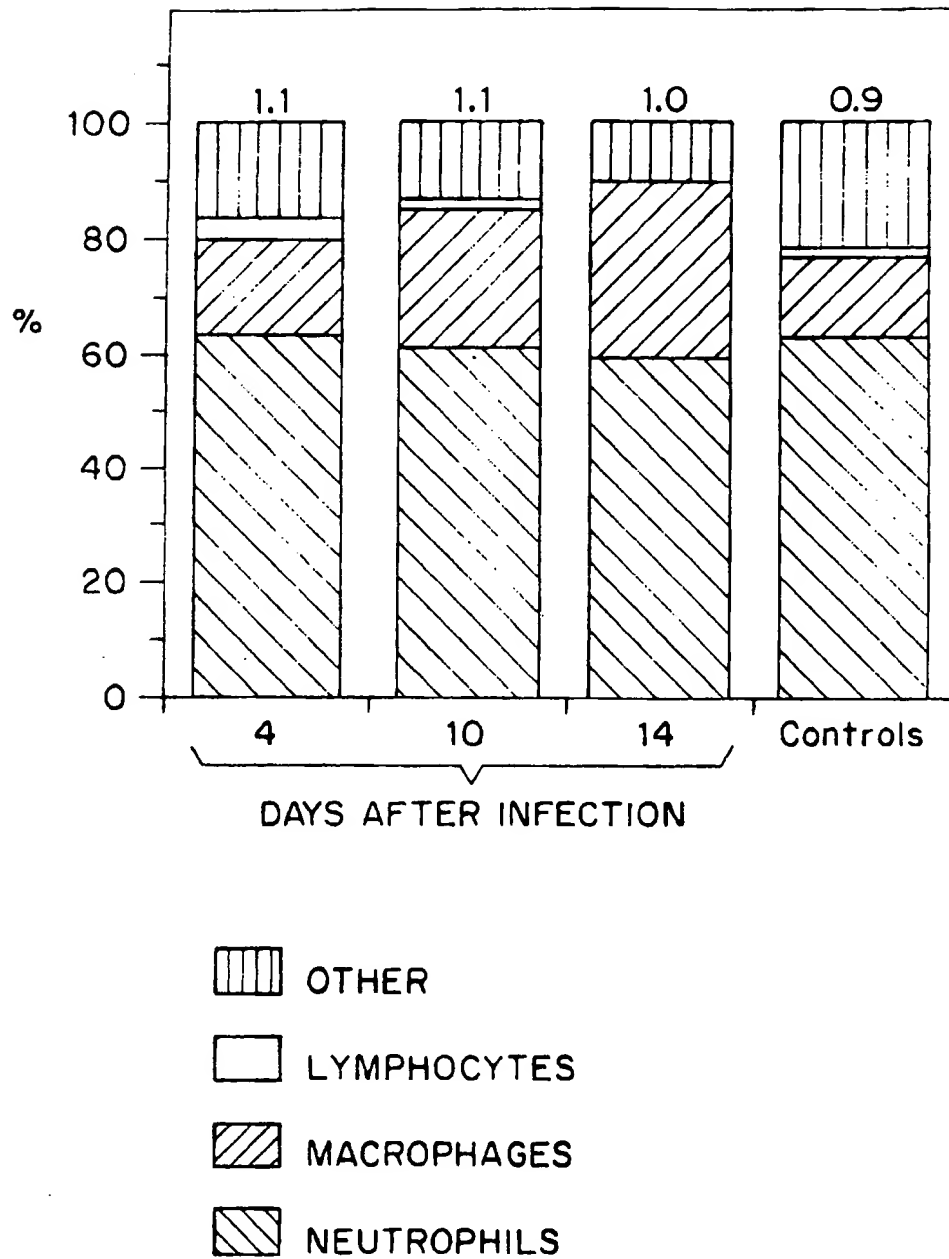


FIG. 14

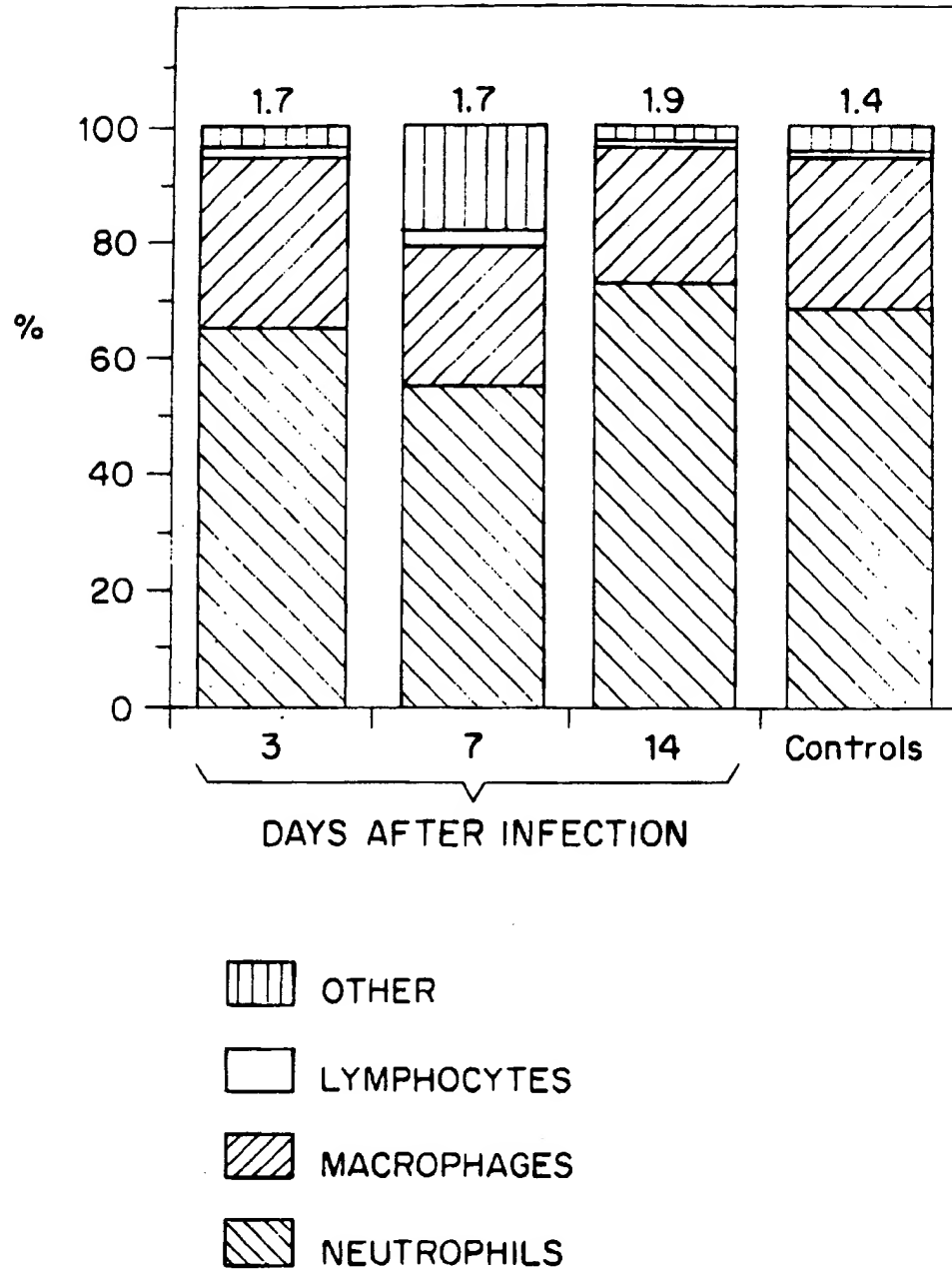
18/56

**FIG. 15**

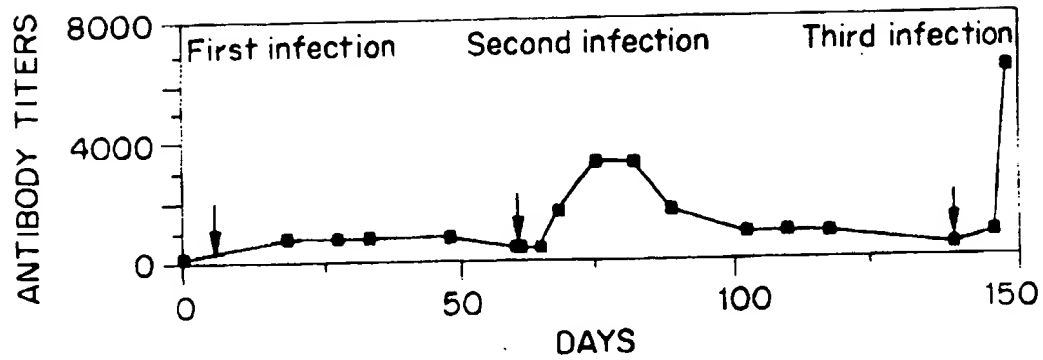
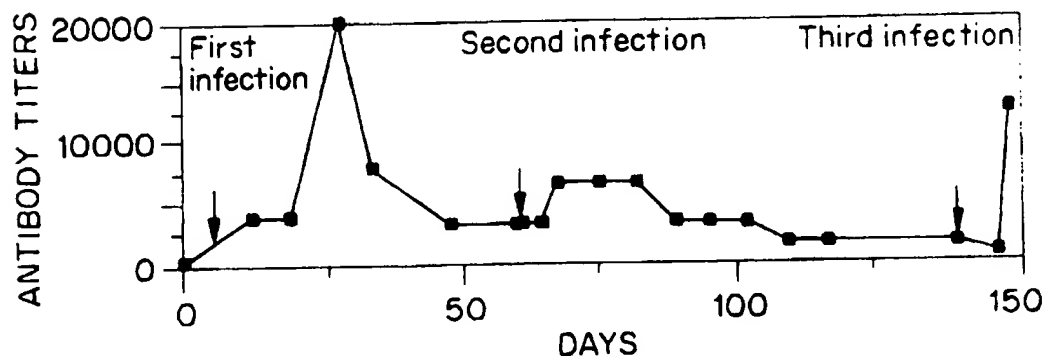
21/56

**FIG. 18A**

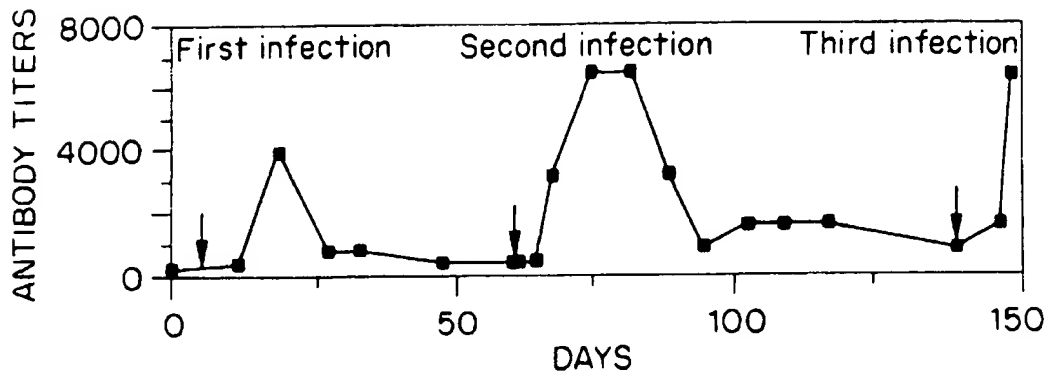
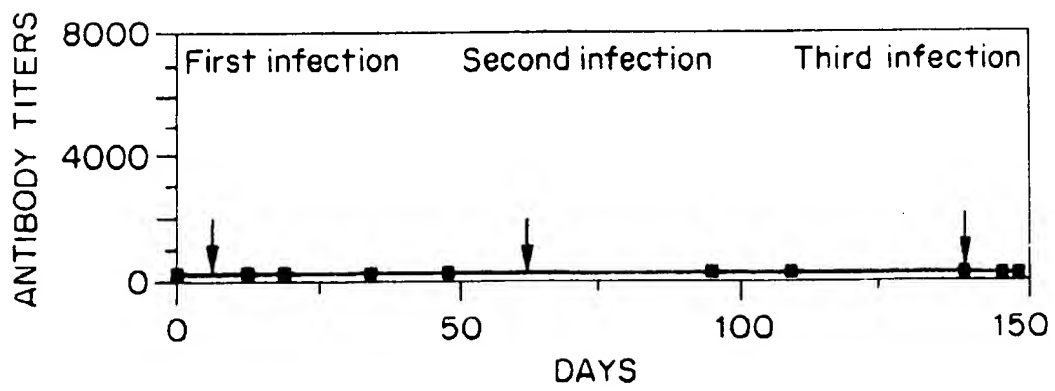
22/56

**FIG. 18B**

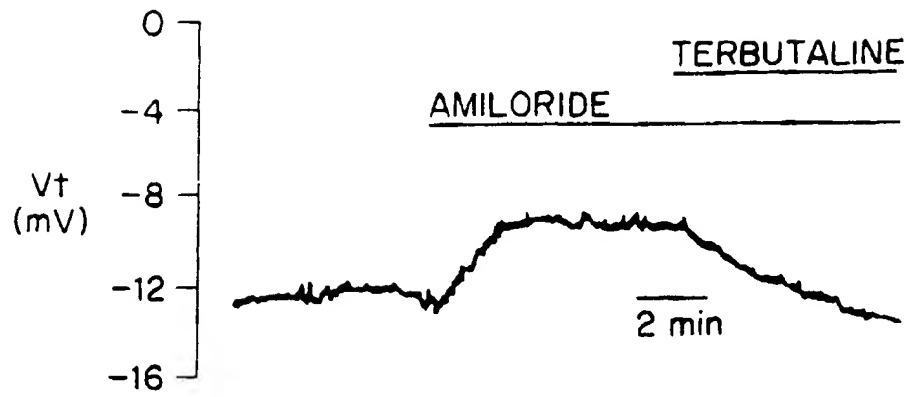
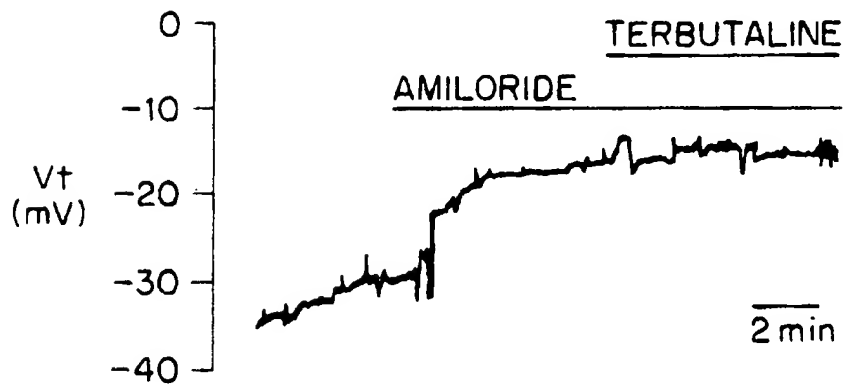
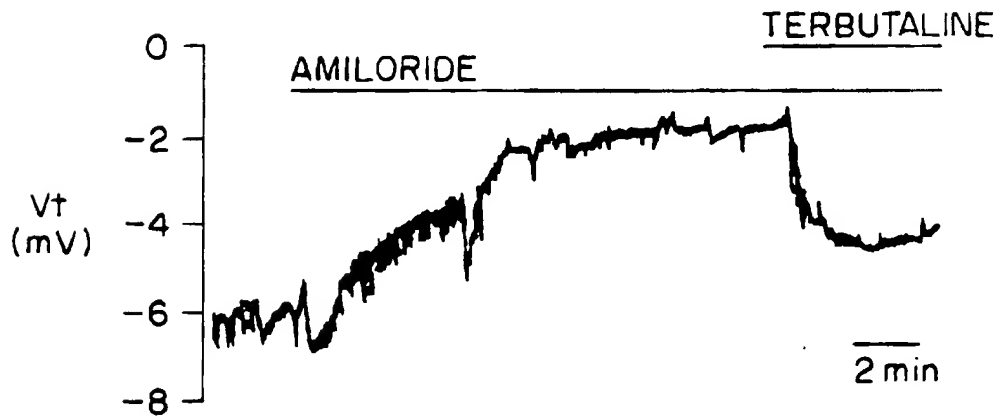
27/56

**FIG. 23A****FIG. 23B**

28/56

**FIG. 23C****FIG. 23D**

32/56

**FIG. 27****FIG. 29A****FIG. 29B**

33/56

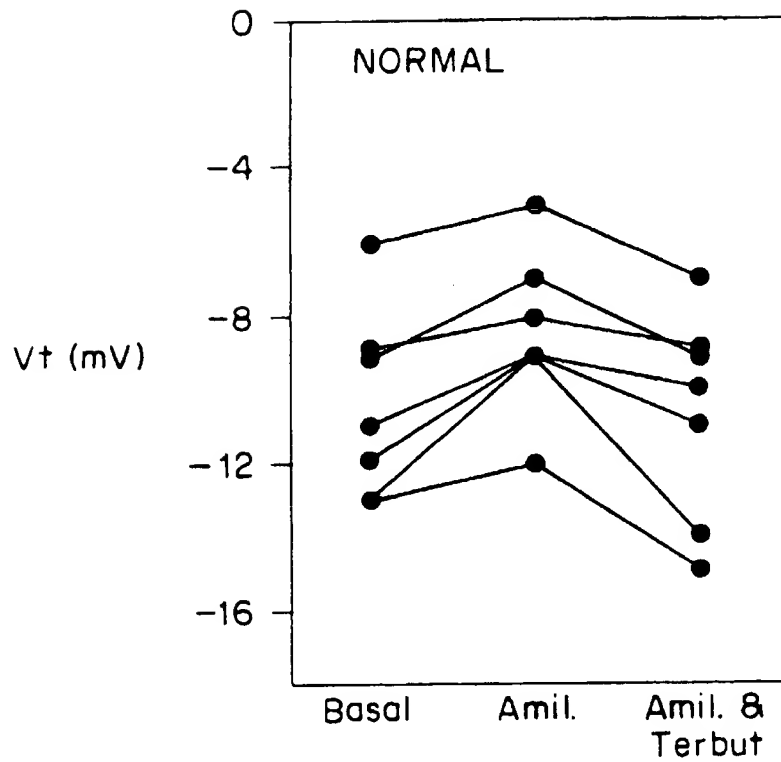


FIG. 28A

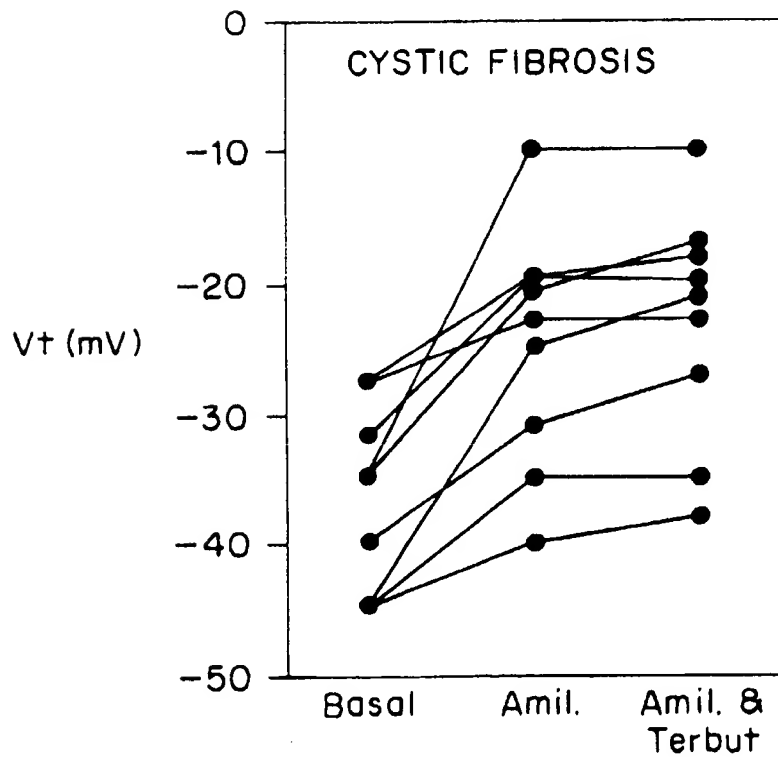
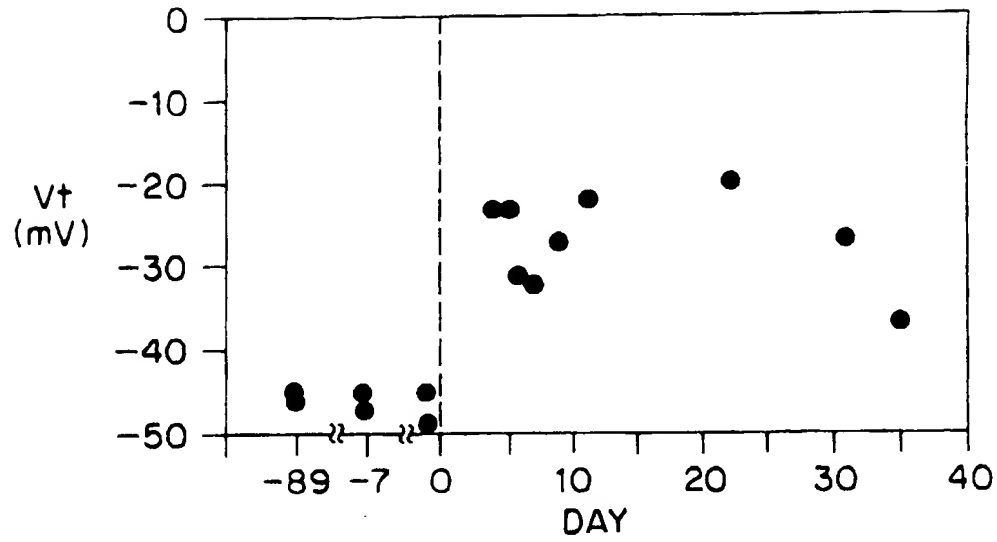
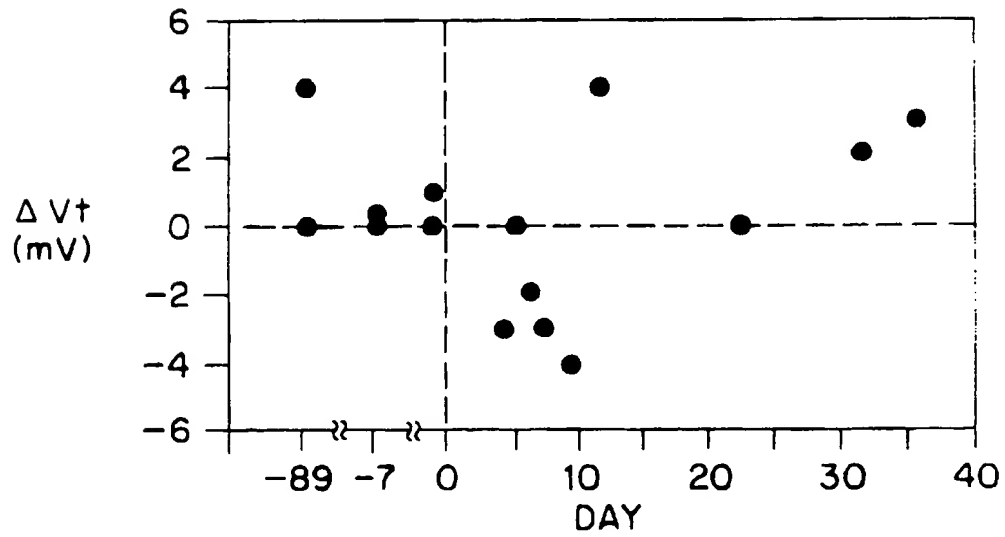


FIG. 28B

34/56

**FIG. 30A****FIG. 30B**

35/56

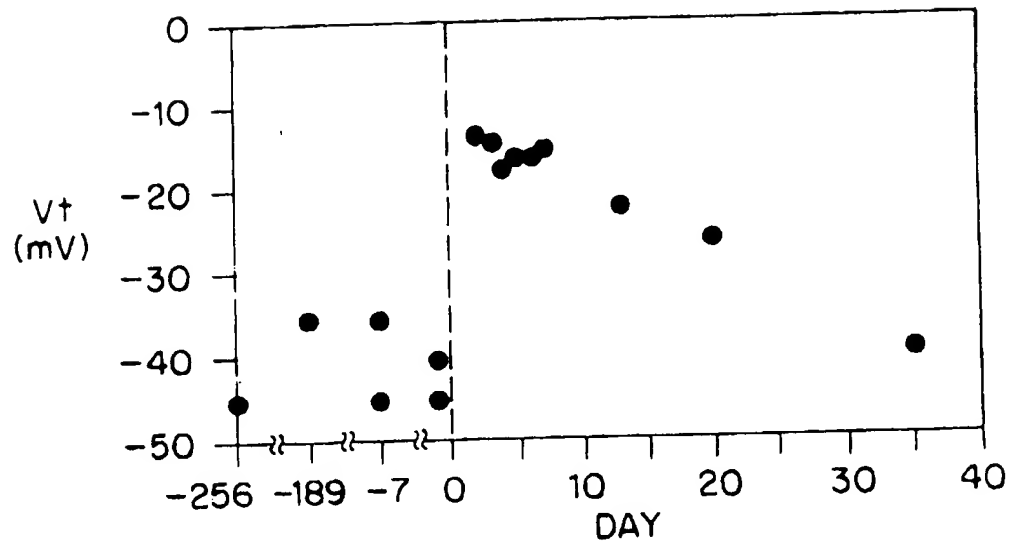


FIG. 30C

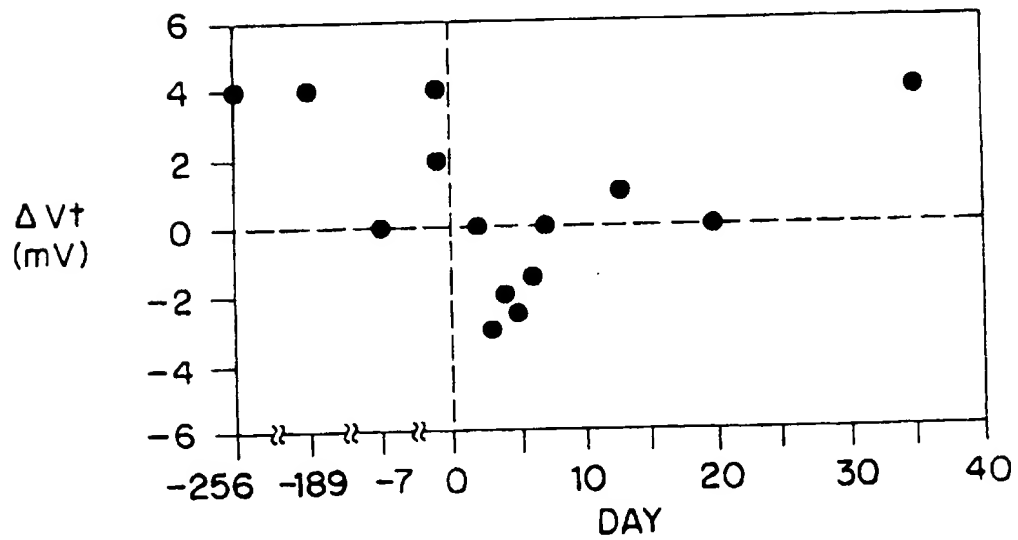
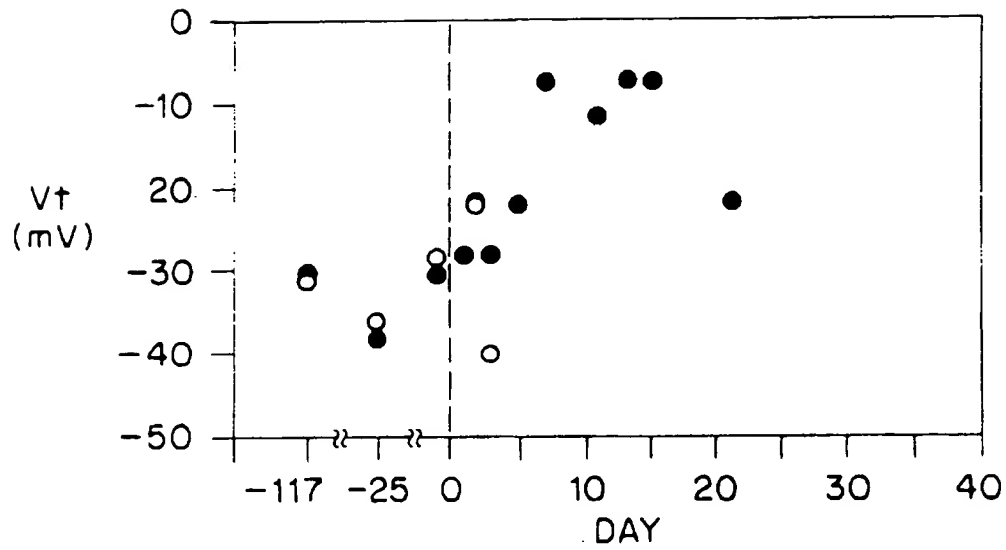
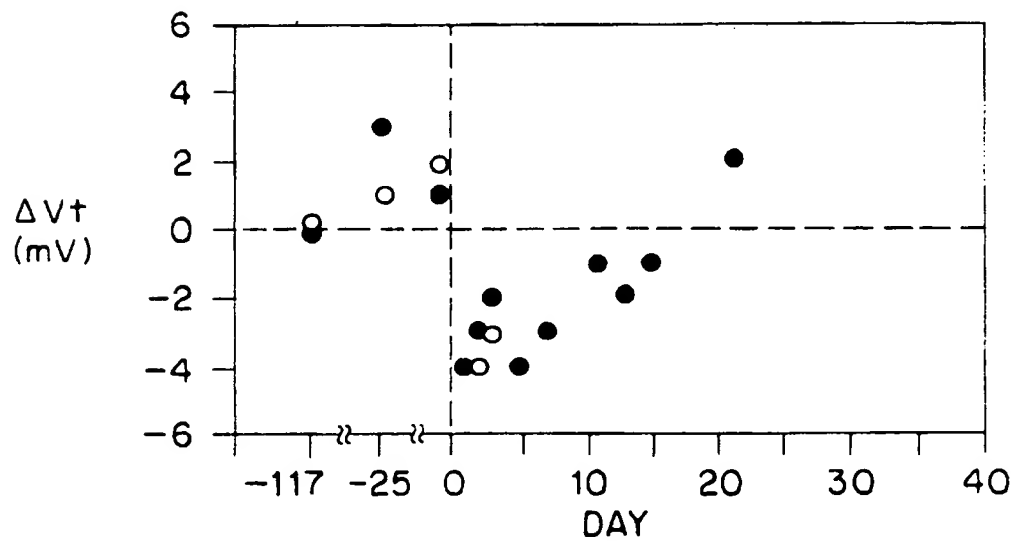
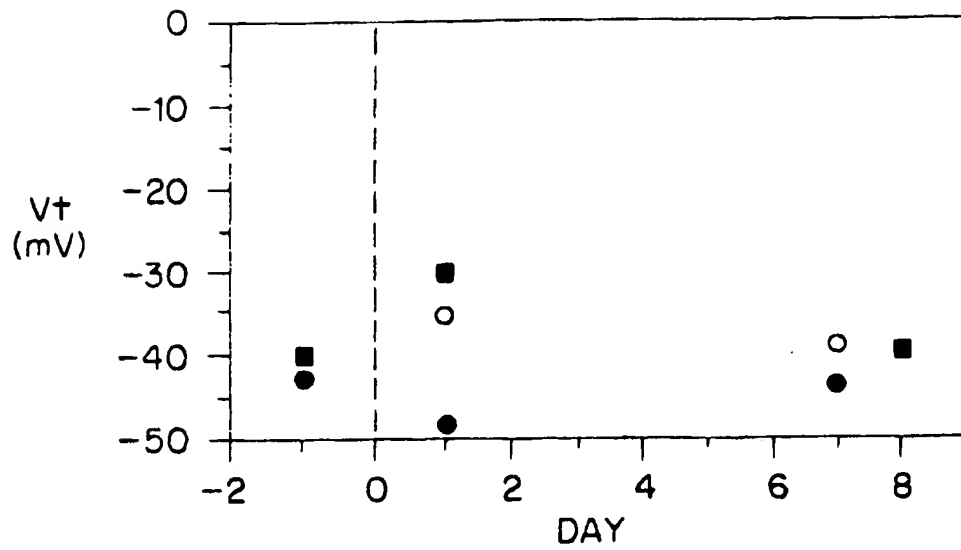
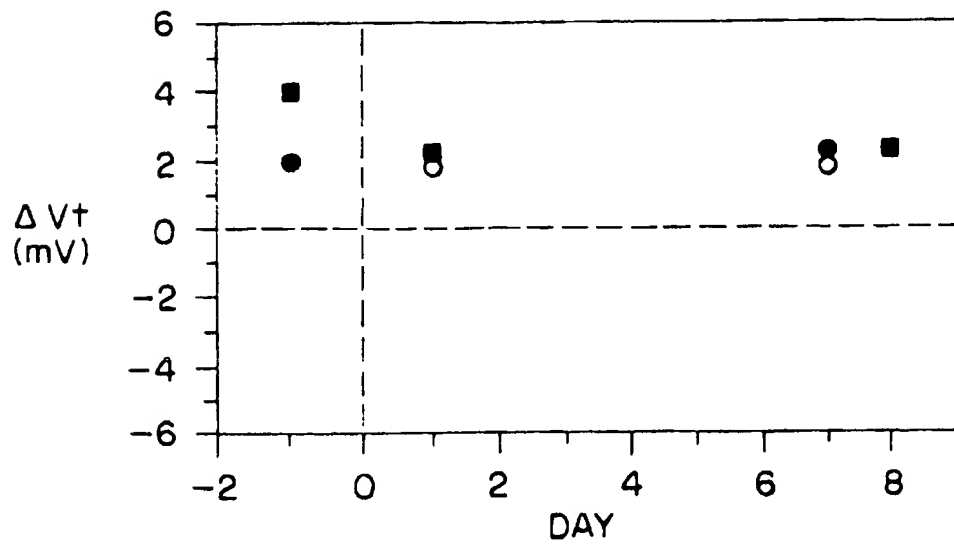


FIG. 30D

36/56

**FIG. 30E****FIG. 30F**

37/56

**FIG. 31A****FIG. 31B**

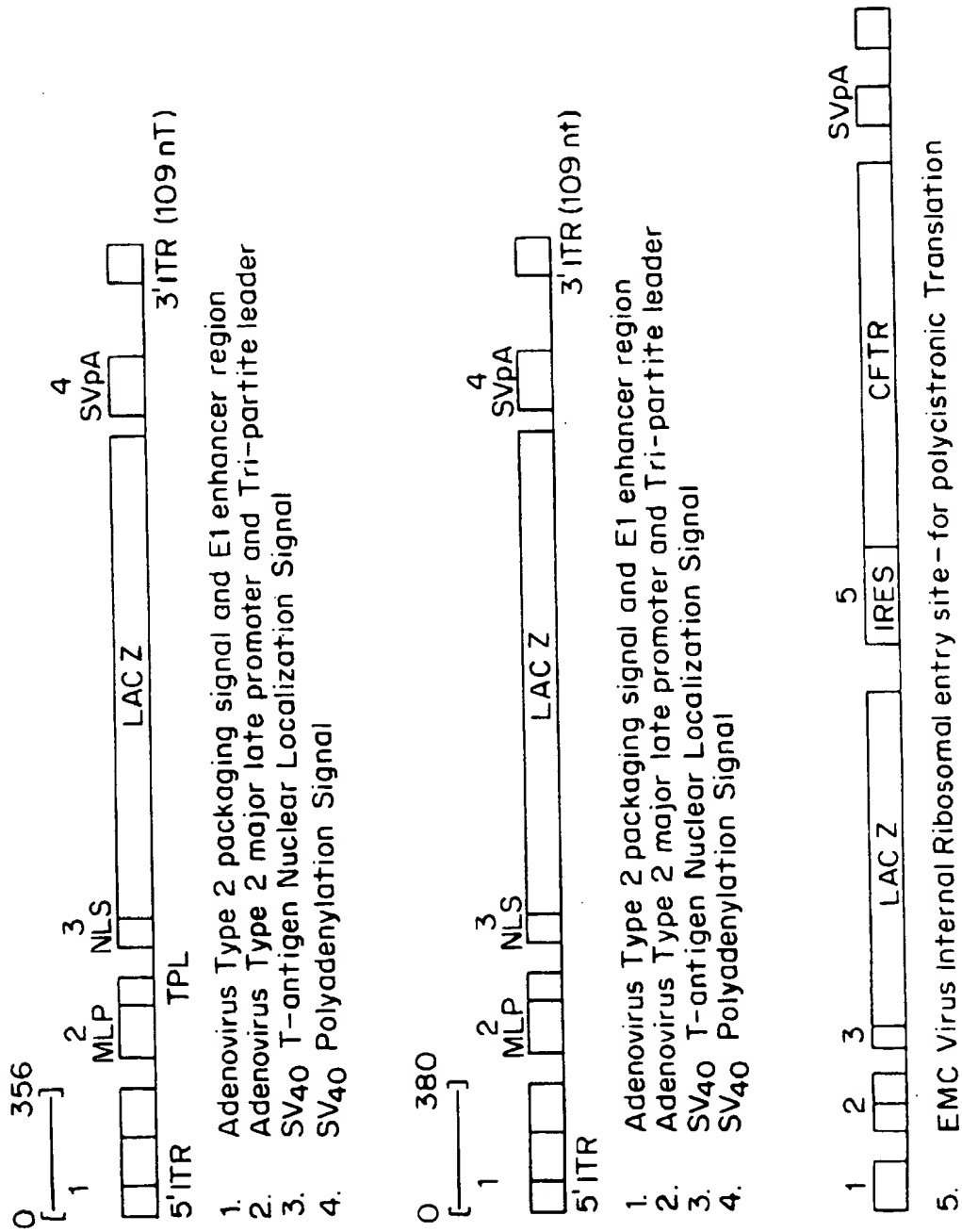
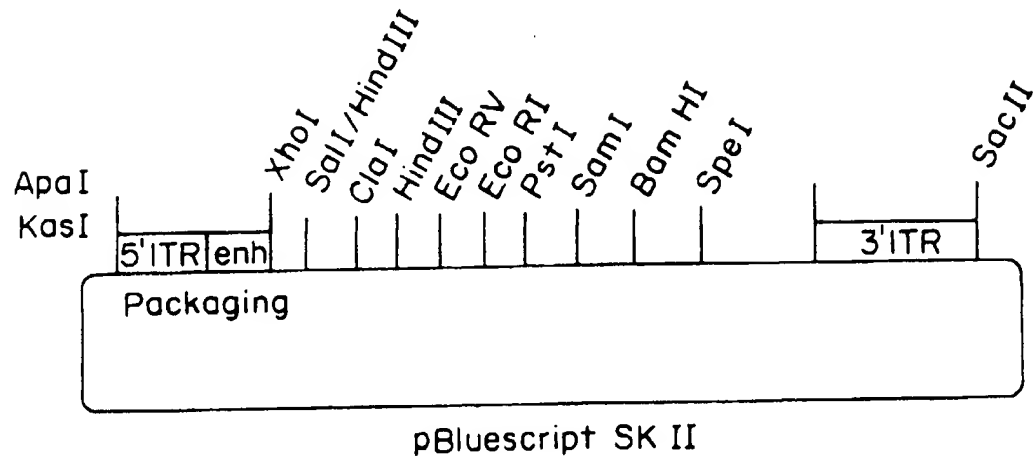


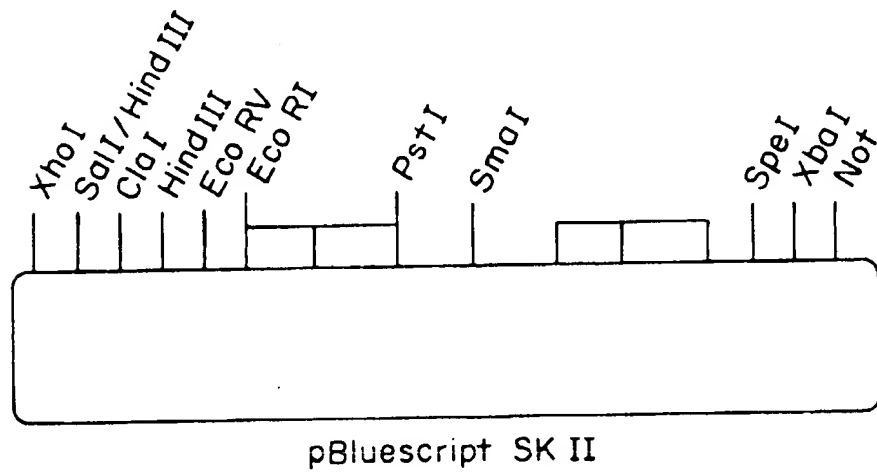
FIG. 32A

39/56

PAV I CLONING CASSETTE



EXPRESSION CASSETTE

**FIG. 32B**

40/56

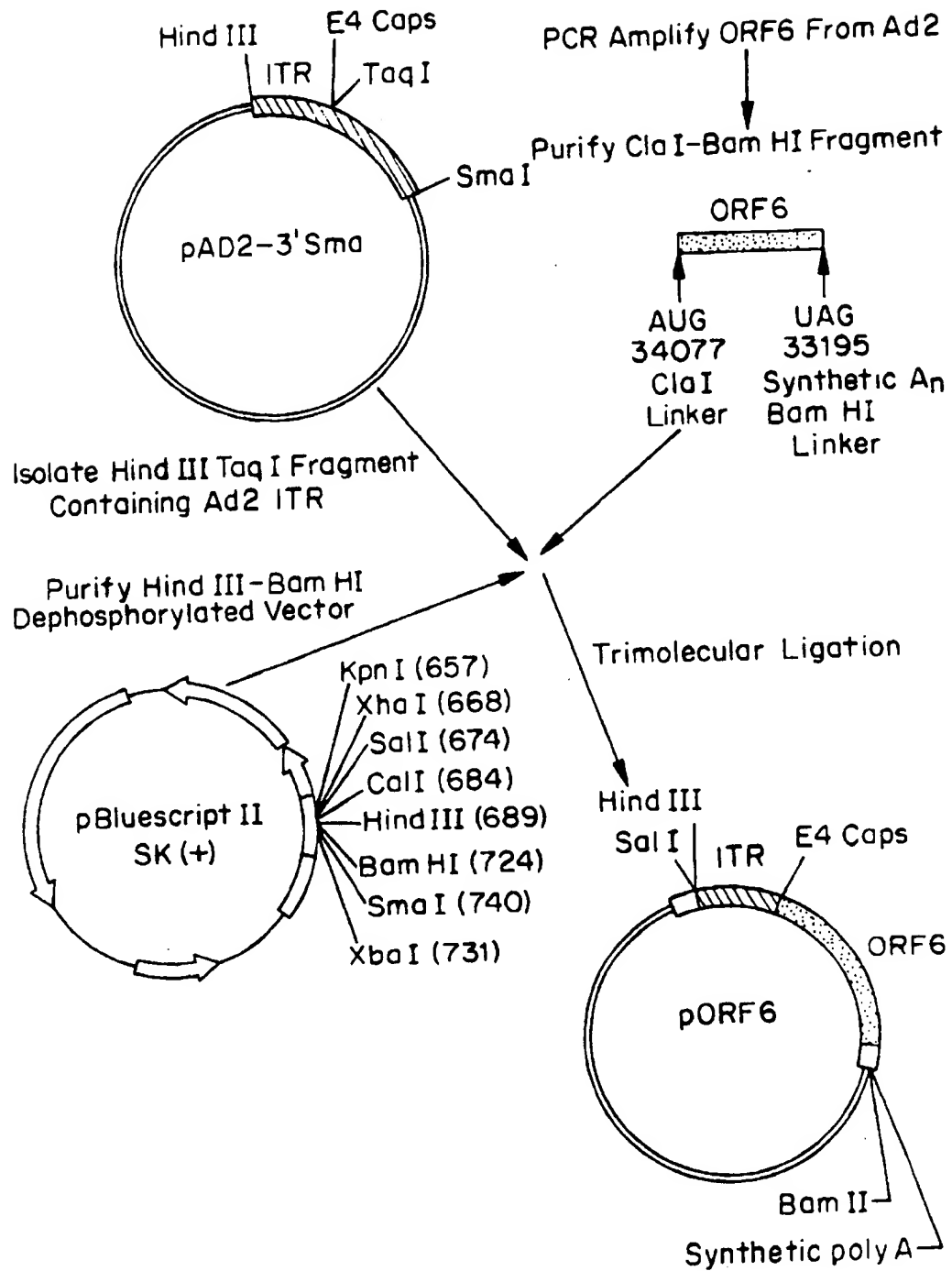
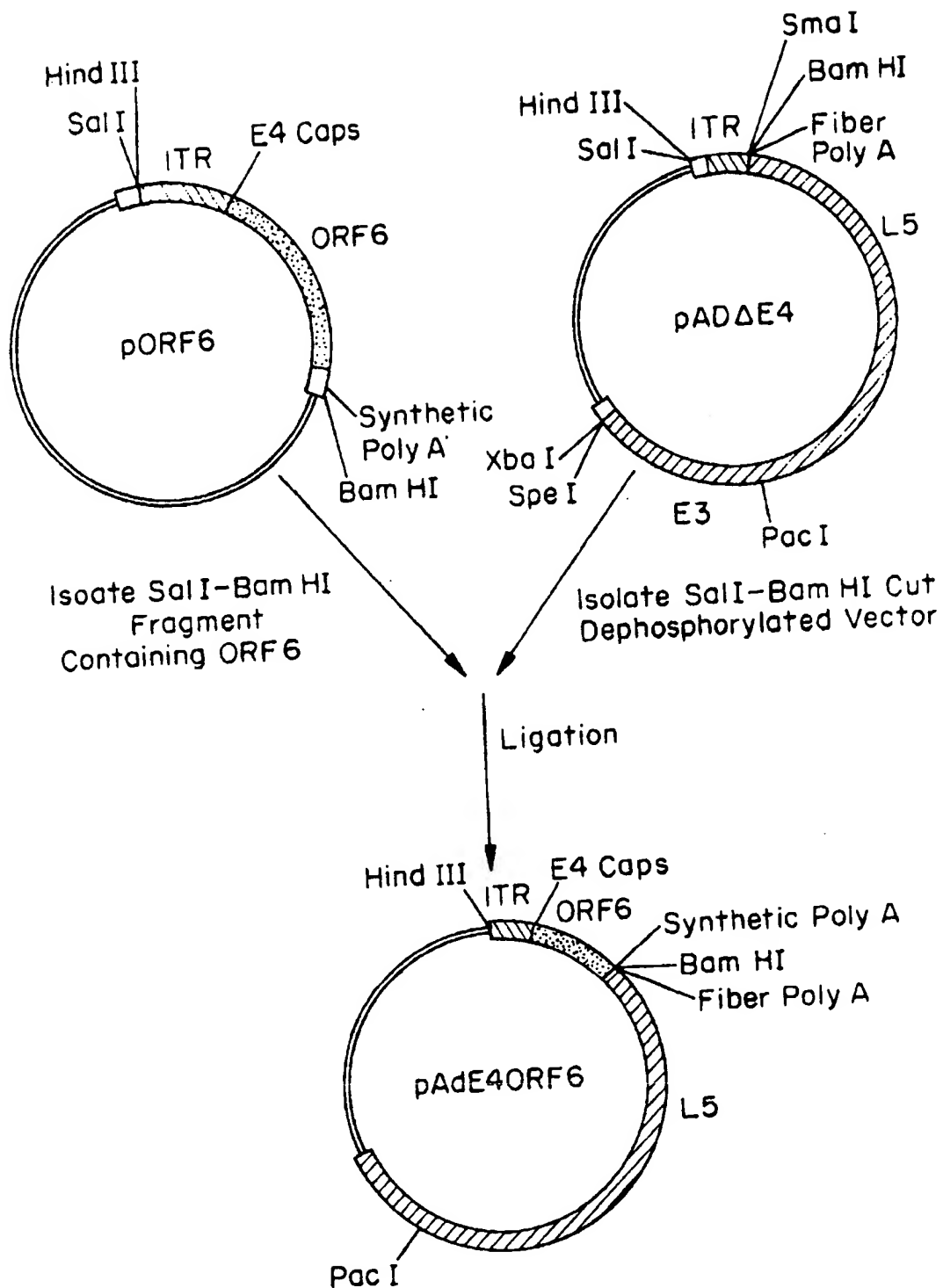
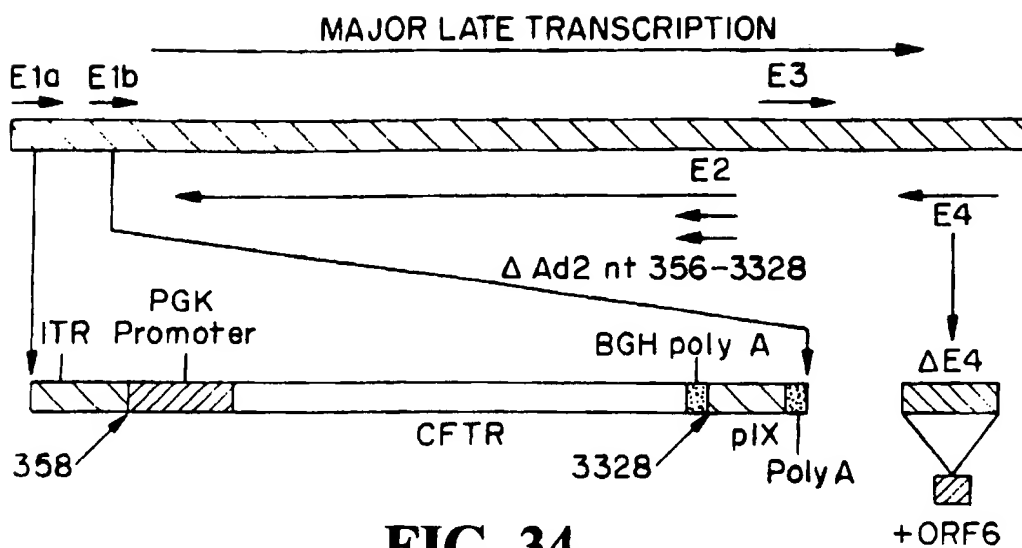
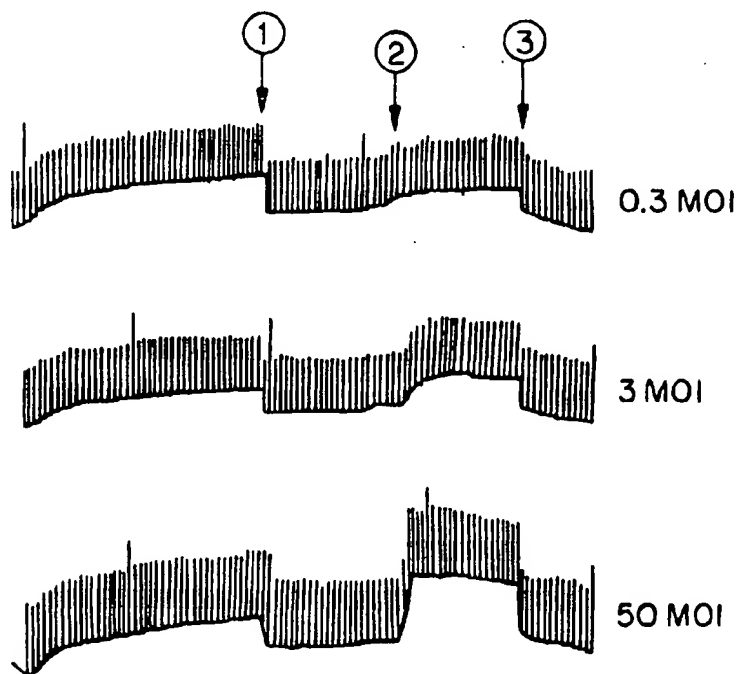


FIG. 33A

41/56

**FIG. 33B**

42/56

**FIG. 34****FIG. 35**

46/56

CLINICAL SIGNS: MONKEY C

AGE 7 YEARS

| DATE | EXAMINATION | HEART | | TEMPERATURE | WEIGHT |
|---------|-------------|-------------|--------------|-------------|--------|
| | | RATE | RESP RATE | | |
| | | (beats/min) | (breath/min) | (Celsius) | (Kg) |
| 5/11/93 | NORMAL | 112 | 16 | 37.8 | 6.4 |
| 5/11/93 | | INFECTION | | | |
| 5/14/93 | NORMAL | 98 | 14 | 38.1 | |
| 5/18/93 | NORMAL | 104 | 16 | 38.3 | |
| 6/4/93 | NORMAL | 108 | 16 | 38.2 | |
| 6/18/93 | NORMAL | 112 | 16 | 38.4 | |
| 6/24/93 | NORMAL | 116 | 18 | 38.8 | |
| 6/24/93 | | INFECTION | | | |
| 6/28/93 | NORMAL | 104 | 18 | 37.9 | |
| 7/5/93 | granulation | 116 | 16 | 37.4 | |
| 7/13/93 | NORMAL | 114 | 20 | 38.3 | |
| 9/17/93 | NORMAL | 108 | 16 | 38.3 | 7 |

FIG. 39A

CLINICAL SIGNS: MONKEY D

AGE 7 YEARS

| DATE | EXAMINATION | HEART | | TEMPERATURE | WEIGHT |
|---------|-------------|-------------|--------------|-------------|--------|
| | | RATE | RESP RATE | | |
| | | (beats/min) | (breath/min) | (Celsius) | (Kg) |
| 5/11/93 | NORMAL | 108 | 18 | 38.3 | 6.25 |
| 5/11/93 | | INFECTION | | | |
| 5/14/93 | NORMAL | 100 | 20 | 38.4 | |
| 5/18/93 | NORMAL | 98 | 20 | 38.4 | |
| 6/4/93 | NORMAL | 106 | 18 | 37.9 | |
| 6/18/93 | NORMAL | 100 | 19 | 38.4 | |
| 6/24/93 | NORMAL | 106 | 16 | 37.8 | |
| 6/24/93 | | INFECTION | | | |
| 6/28/93 | NORMAL | 104 | 16 | 37.4 | |
| 7/5/93 | NORMAL | 102 | 14 | 38.8 | |
| 7/12/93 | granulation | 114 | 16 | 38 | |
| 9/17/93 | NORMAL | 104 | 16 | 38.3 | 6.4 |

FIG. 39B

47/56

CLINICAL SIGNS: MONKEY E

AGE 11 YEARS

| DATE | EXAMINATION | HEART | RESP RATE | TEMPERATURE | WEIGHT |
|---------|-------------|-------------|--------------|-------------|--------|
| | | RATE | | | |
| | | (beats/min) | (breath/min) | (Celsius) | (Kg) |
| 5/11/93 | NORMAL | 120 | 18 | 28.3 | 10 |
| 5/11/93 | | INFECTION | | | |
| 5/14/93 | NORMAL | 112 | 20 | 37.9 | |
| 5/18/93 | NORMAL | 108 | 22 | 38.4 | |
| 6/4/93 | NORMAL | 112 | 20 | 38.3 | |
| 6/18/93 | NORMAL | 106 | 20 | 38.3 | |
| 6/24/93 | NORMAL | 108 | 18 | 38.9 | |
| 6/24/93 | | INFECTION | | | |
| 6/28/93 | NORMAL | 112 | 20 | 38 | |
| 7/5/93 | NORMAL | 106 | 22 | 38.3 | |
| 7/12/93 | NORMAL | 114 | 16 | 38 | |
| 9/17/93 | NORMAL | 114 | 16 | 38.3 | 8.75 |

FIG. 39C

48/56

| CLINICAL LAB RESULTS FROM MONKEY C | | | | | | | | | | |
|------------------------------------|--------|--------|--------|--------|-------|--------|--------|--------|--------|--------|
| DATE | 11-May | 11-May | 14-May | 18-May | 4-Jun | 18-Jun | 24-Jun | 24-Jun | 12-Jul | 17-Sep |
| WBC/mm3 | 6.7 | | | 9 | 8.9 | 7.1 | 7.9 | 7.3 | 10.6 | 8.1 |
| NEUT/mm3 | 1850 | | | 3990 | 3060 | 1480 | 3550 | 3450 | 2210 | 3950 |
| LYMP/mm3 | 4460 | | | 4220 | 4770 | 4780 | 3640 | 2670 | 7270 | 3770 |
| MONO/mm3 | 120 | | | 520 | 600 | 360 | 420 | 550 | 480 | 340 |
| EOS/mm3 | 30 | | | 110 | 190 | 120 | 80 | 400 | 480 | 340 |
| HEMOG, gr/dl | 12.2 | | | 12 | 12.6 | 12.8 | 14 | 13.5 | 13.7 | 13.9 |
| HEMATOCR. % | 38 | | | 38 | 42 | 41 | 45 | 39 | 46 | 43 |
| PLAT k/mm3 | 311 | | | 319 | 343 | 338 | 308 | 281 | 324 | 432 |
| ESR | <1 | | | 1 | 1 | 1 | 0 | <1 | <1 | <1 |
| | | F | I | R | | | S | | O | |
| NA mEq/l | 149 | | | 148 | 147 | | 151 | 147 | 149 | 153 |
| K mEq/l | 3.6 | | | 3.6 | 2.6 | | 3.6 | 3.1 | 3.4 | 3.6 |
| Cl mEq/l | 111 | | | 106 | 107 | | 112 | 108 | 109 | 113 |
| CO ₂ mEq/l | 19 | | | 20 | 20 | | 22 | 21 | 19 | 19 |
| BUN mg/dl | 11 | | | 18 | 11 | | 14 | 13 | 16 | 23 |
| CREAT mg/dl | 1.1 | | | 1 | 1.2 | | 1.1 | 1 | 1.1 | 1.2 |
| GLUCOSE mg/dl | 68 | | | 56 | 81 | | 67 | 87 | 74 | 58 |
| ALB gr/dl | 4.7 | | | 4.3 | 4.7 | | 4.9 | 4.2 | 4.5 | 4.5 |
| T. PROT, gr/dl | 7.3 | | | 6.7 | 7.1 | | 7.4 | 6.9 | 7.1 | 7.4 |
| CALCIUM mg/dl | 10 | | | 9.3 | 9.9 | | 10.2 | 9 | 10.1 | 9.5 |
| PO ₄ mg/dl | 3.3 | | | 5.9 | 5.7 | | 2.9 | 5 | 3.7 | 3.4 |
| ALK. PH IU/l | 117 | | | 376 | 375 | | 117 | 76 | 116 | 164 |
| TOT BIL mg/dl | 0.3 | | | 0.2 | 0.2 | | 0.2 | 0.1 | 0.2 | 0.3 |
| AST IU/l | 38 | | | 37 | 45 | | 28 | 25 | 45 | 34 |
| LDH IU/l | 601 | | | 599 | 740 | | 277 | 406 | 458 | 220 |
| URIC Ac mg/dl | 0.1 | | | 0.1 | <0.1 | | 0.1 | 0.1 | <0.1 | 0.1 |

FIG. 40A

49/56

| CLINICAL LAB RESULTS FROM MONKEY D | | | | | | | | | |
|------------------------------------|--------|--------|--------|--------|-------|--------|--------|--------|---------------|
| DATE | 11-May | 11-May | 14-May | 18-May | 4-Jun | 18-Jun | 24-Jun | 24-Jun | 12-Jul 17-Sep |
| WBC/mm3 | 7 | | 4.2 | 9.9 | 6.7 | 9.1 | 6.9 | 9.4 | 8.3 |
| NEUT/mm3 | 2860 | | 1980 | 3060 | 1090 | 6230 | 1740 | | 3160 |
| LYMP/mm3 | 3660 | | 4180 | 6100 | 4770 | 1820 | 4750 | | 3230 |
| MONO/mm3 | 160 | | 410 | 340 | 500 | 500 | 190 | | 670 |
| EOS/mm3 | 50 | | 150 | 210 | 110 | 240 | 130 | | 210 |
| HEMOG, gr/dl | 10.9 | | 13.7 | 14.7 | 13.6 | 13.9 | 13.6 | | 14.5 |
| HEMATOCR. % | 35 | | 42 | 49 | 44 | 43 | 43 | 44 | 47 |
| PLAT k/mm3 | 268 | | 277 | 413 | 369 | 265 | 300 | 284 | 348 |
| ESR | 1 | | 2 | <1 | 1 | 0 | <1 | <1 | <1 |
| F I R S T | | | | | | | | | |
| NA mEq/l | 147 | | 150 | 150 | | 149 | 147 | 148 | 148 |
| K mEq/l | 3.5 | | 3.5 | 3.6 | | 3.5 | 3.4 | 3.5 | 3 |
| Cl mEq/l | 109 | | 106 | 110 | | 111 | 108 | 109 | 109 |
| CO ₂ mEq/l | 19 | | 20 | 20 | | 23 | 20 | 19 | 16 |
| BUN mg/dl | 19 | | 18 | 20 | | 10 | 16 | 18 | 12 |
| CREAT mg/dl | 1.1 | | 1 | 1.1 | | 1.1 | 1 | 1 | 1 |
| GLUCOSE mg/dl | 65 | | 81 | 72 | | 92 | 78 | 66 | 88 |
| ALB gr/dl | 4.3 | | 4.7 | 5.2 | | 4.2 | 4.6 | 4.5 | 4.7 |
| T. PROT, gr/dl | 6.6 | | 7.4 | 7.8 | | 6.8 | 6.8 | 7.1 | 7.6 |
| CALCIUM mg/dl | 9.3 | | 10.1 | 10.4 | | 9.6 | 9 | 10.3 | 9.5 |
| PO ₄ mg/dl | 6.2 | | 3.5 | 3.6 | | 2.8 | 5 | 5.6 | 4.7 |
| ALK. PH IU/l | 426 | | 104 | 116 | | 82 | 337 | 328 | 101 |
| TOT BIL mg/dl | 0.1 | | 0.3 | 0.2 | | 0.2 | 0.1 | 0.1 | 0.2 |
| AST IU/l | 29 | | 32 | 103 | | 55 | 27 | 25 | 21 |
| LDH IU/l | 520 | | 496 | 912 | | 768 | 615 | 252 | 227 |
| URIC Ac mg/dl | 0.1 | | <0.1 | <0.1 | | 0.1 | 0.1 | <0.1 | 0.1 |
| S E C O N D I N F E C T I O N | | | | | | | | | |

FIG. 40B

FIG. 40C

51/56

CYTOLOGY: MONKEY C

| DATE | 5/11/93 | 5/11/93 | 5/18/93 | 6/4/93 | 6/18/93 | 6/24/93 | 6/24/93 | 6/28/93 | 9/17/93 |
|--------------|---------|---------|---------|--------|---------|---------|---------|---------|---------|
| LEFT NOSTRIL | | | | | | | S | B | 69 |
| Sq. Epith. | 68 | F | 78 | 63 | 72 | 74 | E | I | 30 |
| Resp. Epith. | 30 | I | 18 | 34 | 24 | 25 | C | O | 0 |
| Neutrophils | 1 | R | 2 | 3 | 2 | 0 | O | P | 0 |
| Lymphocytes | 1 | S | 2 | 0 | 1 | 1 | N | S | 0 |
| Eosinophils | 0 | T | 0 | 0 | 1 | 0 | D | Y | 1 |

FIG. 41A

CYTOLOGY: MONKEY D

| DATE | 5/11/93 | 5/11/93 | 5/18/93 | 6/4/93 | 6/18/93 | 6/24/93 | 6/24/93 | 7/5/93 | 9/17/93 |
|--------------|---------|---------|---------|--------|---------|---------|---------|--------|---------|
| LEFT NOSTRIL | | | | | | | S | B | 73 |
| Sq. Epith. | 60 | F | 60 | 72 | 72 | 84 | E | I | 25 |
| Resp. Epith. | 39 | I | 39 | 26 | 25 | 14 | C | O | 2 |
| Neutrophils | 1 | R | 1 | 0 | 1 | 2 | O | P | 0 |
| Lymphocytes | 0 | S | 2 | 2 | 1 | 0 | N | S | 0 |
| Eosinophils | 0 | T | 0 | 0 | 1 | 0 | D | Y | 0 |

FIG. 41B

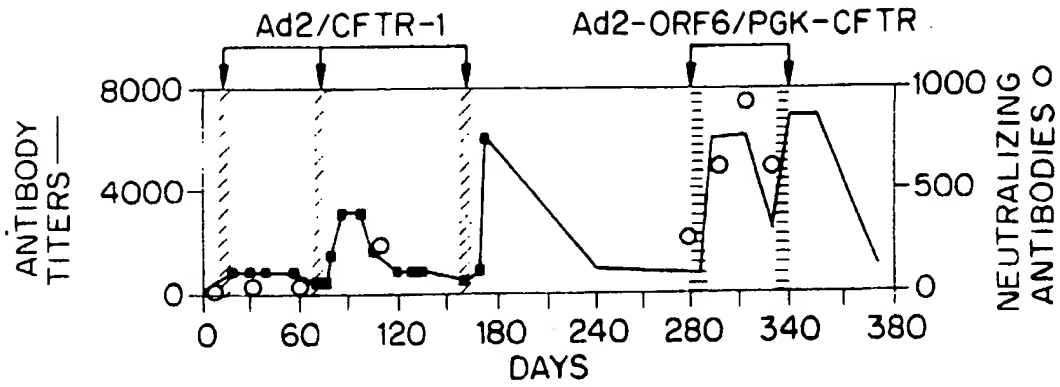
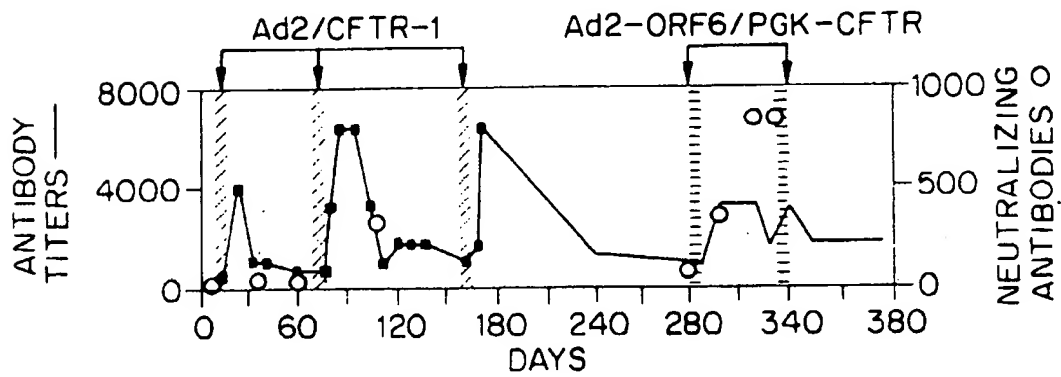
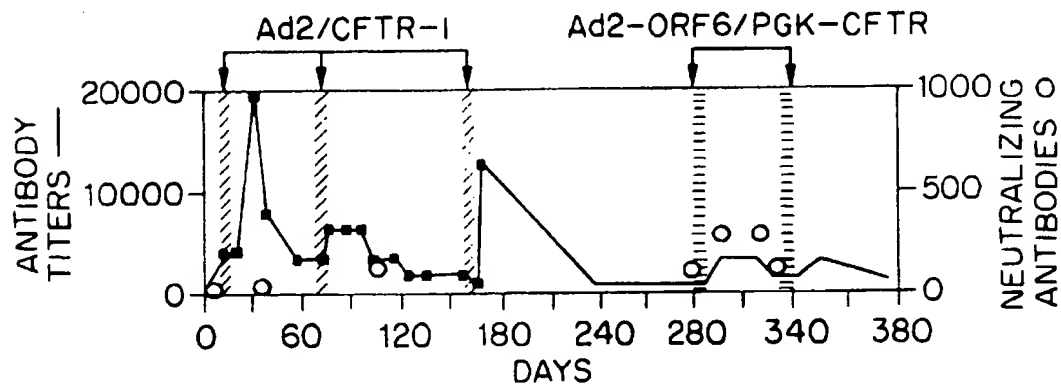
52/56

CYTOLOGY: MONKEY E

| DATE | 5/11/93 | 5/11/93 | 5/18/93 | 6/4/93 | 6/18/93 | 6/24/93 | 6/24/93 | 7/12/93 | 9/17/93 |
|--------------|---------|---------|---------|--------|---------|---------|---------|---------|---------|
| LEFT NOSTRIL | | | | | | | | | |
| Sq. Epith. | 60 | F | 60 | 72 | 72 | 84 | S | B | 73 |
| Resp. Epith. | 39 | I | 39 | 26 | 25 | 14 | E | I | 25 |
| Neutrophils | 1 | R | 1 | 0 | 1 | 2 | C | O | 2 |
| Lymphocytes | 0 | S | 2 | 2 | 1 | 0 | O | P | 0 |
| Eosinophils | 0 | T | 0 | 0 | 1 | 0 | N | S | 0 |
| | | | | | | | D | Y | |

FIG. 41C

56/56

**FIG. 45A****FIG. 45B****FIG. 45C**